UNIVERSITY OF ALBERTA

DEVELOPMENT OF STRATEGIES TO OVERCOME LIMITATIONS TO FUNCTIONAL RECOVERY AFTER PERIPHERAL NERVE INJURIES

BY

OLAWALE A.R.SULAIMAN ©

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UNIVERSITY OF ALBERTA

FACULTY OF GRADUATE STUDIES AND RESEARCH

The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled DEVELOPMENT OF STRATEGIES TO OVERCOME LIMITATIONS TO FUNCTIONAL RECOVERY AFTER PERIPHERAL NERVE INJURIES, submitted by OLAWALE ADENIYI RAFIU SULAIMAN in partial fulfillment of the requirements for the degree of DOCTOR OF PHILOSOPHY

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DEDICATION

This thesis is dedicated to my parents who encouraged and supported my educational ambitions as a child, despite the fact that they did not have any western education themselves. I also dedicate this thesis to my fiancee, Patricia Ugoalah, for being my best friend and for her endless love, support and understanding.

ABSTRACT

Nerve regeneration after peripheral nerve injuries is relatively better than after injuries to the central nervous system. The difference in the regenerative capacity is attributed to the provision of a growth-supportive environment by the Schwann cells of the of the peripheral nervous system. However, functional recovery after peripheral nerve injuries is often very poor despite the regenerative capacity. Factors limiting functional recovery are unknown, and tremendous advancements in the microsurgical repair of injured nerves have not made significant improvement in functional outcome after nerve injuries. Hence, using rat models of nerve injury and repair, the objectives of this thesis were, 1) to study some of the cell-molecular mechanisms of poor functional recovery after peripheral nerve injuries, and 2) to develop experimental strategies to promote functional recovery. We studied the progressive changes in the capacity of injured motoneurons to regenerate axons under conditions that mimic the pathophysiology of nerve injuries in humans (i.e. after immediate and delayed nerve repairs), and how these changes relate to the functional state of the Schwann cells of the distal nerve stumps. The effects of transforming growth factor-beta (TGF-β) on the capacity of chronically denervated Schwann cells to support motor axonal regeneration and that of the immunophilin, FK506, to promote motor axonal regeneration after delayed nerve repair, were explored as possible strategies to promote functional recovery after nerve injuries.

Adult male Sprague-Dawley rats were used in all experiments under aseptic conditions. The common peroneal (CP) and tibial (TIB) branches of rat sciatic nerve were used in a cross-suture paradigm of nerve injury and repair. Briefly, the CP and TIB nerves were cut and either immediate or delayed TIB-CP cross-suture was performed to allow regeneration of TIB motoneurons into freshly or chronically denervated CP nerve stumps. Direct neuroanatomical estimation of the numbers of TIB motoneurons that regenerated axons was carried out using fluorescent neurotracers (Fluorogold or Fluororuby) to backlabel TIB neuronal cell bodies. Also, numbers of regenerated axons were counted and their myelination by the SCs was examined histomorphologically. Reverse-transcriptase polymerase chain reaction was used to determine changes in gene expression of SCs.

The major findings of this thesis include i) delayed nerve repair (> 4weeks) leads to progressive deterioration of the capacity of the Schwann cells of the distal nerve stumps to support motor axonal regeneration; ii) this declining capacity of Schwann cells to support motor axonal regeneration is due, at least in part, to the progressive downregulation of the expression of glial-derived neurotrophic factor; iii) 48 hour *in vitro* incubation of chronically denervated Schwann cells with TGF-β dramatically improved their capacity to support motor axonal regeneration *in vivo*; and iv) FK506 increased the rate of axonal regeneration and the number of motoneurons that regenerated axons, even after delayed nerve repair. In conclusion, the results of this thesis demonstrate that poor functional recovery after nerve injuries is primarily due to the detrimental effect of delayed reinnervation of the Schwann cells of the distal stumps of injured nerves, since they lose their capacity to support motor axonal regeneration. However, these detrimental effects of delayed nerve repair are reversible by cytokines such as TGF-β, and preventable by FK506 which accelerates motor axonal regeneration and thereby, promotes timely reinnervation of Schwann cells.

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LIST OF FREQUENTLY USED ABBREVIATIONS

Brain-derived Neurotrophic Factor (BDNF)

Calcitonin Gene-related Peptide (CGRP)

Central Nervous System (CNS)

Dorsal Root Ganglion (DRG)

Glial-derived Neurotrophic Factor (GDNF)

Growth-associated Protein-43 (GAP-43)

Myelin Basic Protein (MBP)

Myelin-associated Glycoprotein (MAG)

Nerve Growth Factor (NGF)

Neural-cell Adhesion Molecules (N-CAM)

Neurotrophin Receptor (p75NTR)

Neurotrophin-4/5 (NT-4/5),

Neurotrophin-6 (NT-6)

Neurotrpphin 3 (NT-3)

Peripheral Nervous System (PNS)

Protein Zero (P_0)

Regeneration Associated Genes (RAGS)

Schwann Cell (SC)

Transforming Growth Factor-β (TGF-β)

Tropomyosin Receptor Kinase-B (TrkB)

CHAPTER 1

INTRODUCTION AND STUDY OBJECTIVES: PERIPHERAL NERVE INJURIES

1.0 INTRODUCTION

Mammalian central nervous system (CNS) neurons are virtually incapable of regenerating axons after injury in contrast to their peripheral nervous system (PNS) counterparts which do regenerate, albeit slowly and incompletely (Cajal, 1928; Kiernan, 1979; reviewed by Dezawa, 2000). The non-permissive environment of the CNS has been attributed to the prolonged persistence of growth-inhibitory myelin-derived proteins such as NI-35/NI-250/Nogo-A, myelin-associated glycoprotein (MAG) (Schnell & Schwab, 1990; George & Griffin, 1994; Mckerracher et al., 1994; Mukhopadhyay et al., 1994; Bandtlow & Schwab, 2000; Chen et al., 2000; Grandpre et al., 2000) subsequent to the inhibition of the phagocytic activity of macrophages and/or microglia by macrophage inhibitory factor (Hirschberg & Schwartz, 1995) and other growth-inhibitory components such as tenascin-C, proteoglycans including chondroitin-, heparan- and keratan sulphate proteoglycan (Muller, 1993; Lips et al., 1995; Muller et al., 1996) as well as the growth-inhibitory environment created by the myelin-forming cells, the oligodendrocytes (Lazarov-Spiegler et al., 1998; Qui et al., 2000).The myelinating glial cell of the PNS is the Schwann cell (SC) which, in contrast to the oligodendrocyte in the CNS, provides a growth-permissive environment for axonal regeneration in both the PNS and the CNS, particularly in association with phagocytic macrophages which infiltrate denervated distal nerve stumps to phagocytose the myelin of denervated SCs (David & Aguayo,1981; Perry et al, 1987; Keirstead et al., 1989; Chen & Bisby, 1993; Zeer-Brann et al.,1998; Weinstein, 1999; Popovic et al., 2000).

However, despite the capacity for axonal growth in the PNS and the permissive environment provided by the SCs of the distal stumps of injured peripheral nerves, functional recovery is often disappointing after PNS injury even with microsurgical repair. In order to attain any form of functional recovery after peripheral nerve injury, axotomised neurons must i) regrow the damaged axon, ii) upregulate and maintain the upregulation of the required regeneration associated genes (RAGs e.g. GAP-43, tubulin, actin), and transcription factors such as c-fos, c-jun and KROX 24 all of which have been associated with axonal regeneration, iii) continue axonal regrowth through the lesion site (i.e. overcome inflammation-induced scar at the lesion site), iii) elongate the axons in the correct direction (correct endoneurial tubes), iv) topographically reinnervate their original target (target reinnervation), and v) restore normal electrophysiological properties (Freed et al., 1985; Bisby & Tetzlaff, 1992; Jenkins et al., 1993; Robinson, 1994; Hayes et al., 1995; Aubert et al., 1995; Yen & Kalb, 1995; Benowitz and Routtenberg, 1997; Herdegen et al., 1997; Fu & Gordon, 1997; Terenghi, 1999). Successful completion of all these steps in the process of nerve regeneration require a well-coordinated and time-related changes in gene expression of the injured neurons, the SCs of the distal nerve stumps and the denervated muscles. Inadequacies in any of the steps of the regenerative process may lead to poor functional recovery.

Both CNS and PNS neurons can initiate the initial process of axonal regrowth by sending out axonal sprouts from the proximal stump of injured nerves, but only the PNS neurons can accomplish the other steps in the regenerative process as CNS neurons either fail to upregulate RAGs or do so at very low levels and, CNS initial effort to regrow axons aborts within the inhibitory environment of the denervated oligodendrocytes in the scar tissue (Schwab & Bartholdi, 1996; Chen et al., 2000). Despite the seemingly excellent regenerative capacity of the PNS, clinical experience has established that functional recovery is often poor, particularly for injuries which sever large nerves such as brachial and lumbar plexus nerve trunks (Sunderland, 1978; MacKinnon, 1989; Millesi, 1990; Terzis & Smith, 1990; Fu & Gordon, 1997;Gordon & Fu 1997; Kline, 2000; Allan, 2000). This is because of the slow rate of regeneration (1-3mm/day) of injured neurons results in their chronic axotomy and chronic denervation of the SCs of the distal nerve stumps, during which RAGs expression by these neurons may be lost and the growth-permissive environment in the distal nerve stumps may progressively deteriorate. Another important factor which may further exacerbate the limited functional recovery after nerve injuries is the misdirection of many regenerating axons into the wrong endoneurial tubes and their reinnervation of inappropriate targets (Sunderland, 1978; Fu & Gordon, 1997). Hence, challenges to achieving successful axonal regeneration are i) achieving nerve regeneration at a faster rate, ii) maintaining the growth-supportive environment of the distal nerve stumps while neurons regenerate their axons and iii) directing regenerating axons into the appropriate endoneurial tubes. This thesis focuses on understanding these factors that lead to poor functional recovery and experimental strategies to overcome them.

1.1 ETIOLOGY (COMMON CAUSES) AND EPIDEMIOLOGY OF PERIPHERAL NERVE INJURIES

Peripheral nerve injuries are relatively common affecting 5% of trauma population (Noble at al., 1998). The proportion of trauma population affected by peripheral nerve injuries may still be underestimated since with improved trauma services, more cases of peripheral nerve injuries are being recognised. It is essential to adopt better, more consistent and systematic evaluation of the trauma population for quick identification of patients afflicted with peripheral nerve injuries that are likely to benefit from microsurgical repair, which remains the keystone of management of this group of patients (Spinner & Kline, 2000). Secondly, early identification and treatment of peripheral nerve injuries is important since, majority of the trauma population are in the highly reproductive and economically productive age groups (20-30 years old), who if left untreated, become seriously disabled (Midha, 1997; Noble et al., 1998; Allan, 2000).

1.1.1 Incidence and Prevalence of Peripheral Nerve Injuries

Peripheral nerve injuries can result from mechanical (i.e. direct injuries, compression, and entrapment), thermal, chemical, congenital, or pathological etiologies. For simplicity, peripheral nerve injuries are divided into upper extremity and lower extremity peripheral nerve injuries. Upper extremity peripheral nerve injuries are more common than lower extremity injuries, with brachial plexus injuries representing the most common upper extremity injuries, and sciatic nerve injuries representing the most common lower extremity injuries (Midha, 1997; Kline et al., 1998). In a trauma population analysed by Noble et al., (1998) it was found that, generally peripheral nerve injuries are caused by, motor vehicle accidents (MVA, 46%), motorcycle accidents (9.9%), pedestrian accidents (8.6%), gunshot wounds (GSW, 7.4%), fall (6.8%), industrial accidents (6.2%), stab wounds (5.6%), recreational MVA (RMVA; 5.6%), bicycle accidents (1.2%), assault (1.2%) and other causes (2.5%) (Figure 1).

However, analyses of the causes of the most common upper (brachial plexus injuries; Midha, 1997) and lower (sciatic nerve injuries; Kline et al., 1998) extremities PNI showed different patterns. Causes of brachial plexus injuries in a multi-trauma population were as follows, MVA (29%), motorcycle (22%), pedestrian accidents (13%), gunshot wounds (GSW; 11%), industrial accidents (7%), snowmobile (6%), fall (4%), bicycle (4%), stab (2%), and others (2%) (see Figure 2). Causes of sciatic nerve injuries differ depending on whether the injuries were at the buttock or thigh level, although buttock level injuries predominate (60%). Injuries at the buttock level were caused by, injection (59%), fracture/dislocation (13%), blunt contusion (7.8%), GSW (6.0%), hip athrosplastic surgery (5.6%), laceration (4.7%), compression (3.5%) (Figure 3a). Injuries at the thigh level were caused by GSW (36.7%), fracture (23.3%), laceration (17.3%), contusions (16%), compression (4%), iatrogenic (2.7%) (see Figure 3b). It is apparent that majority of the causes of peripheral nerve injuries are, to a large extent, preventable especially causes such as MVA, gunshot wounds, recreational accidents and iatrogenic causes (e.g. injection injuries of the sciatic nerve).

FIGURE 1.1: CAUSES OF PERIPHERAL NERVE INJURIES

(NOBLE ET AL., 1998) .

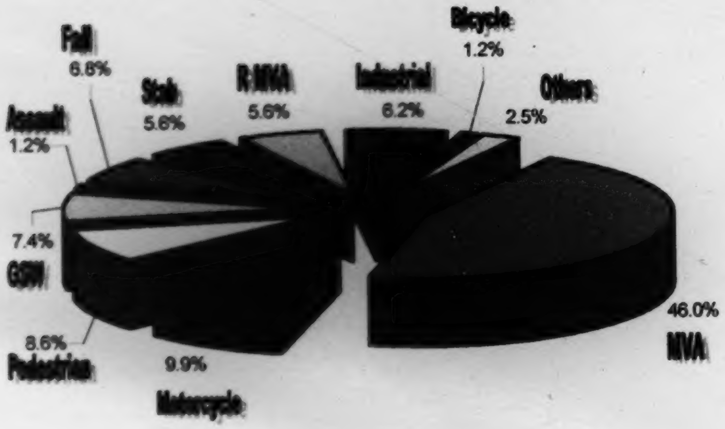
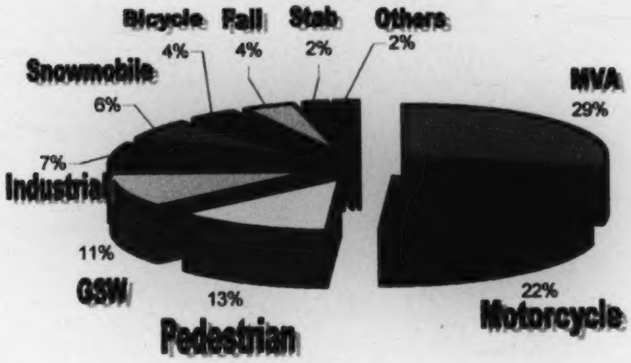


FIGURE 1.2: CAUSES OF BRACHIAL PLEXUS INJURIES
(MIDHA, 1997)



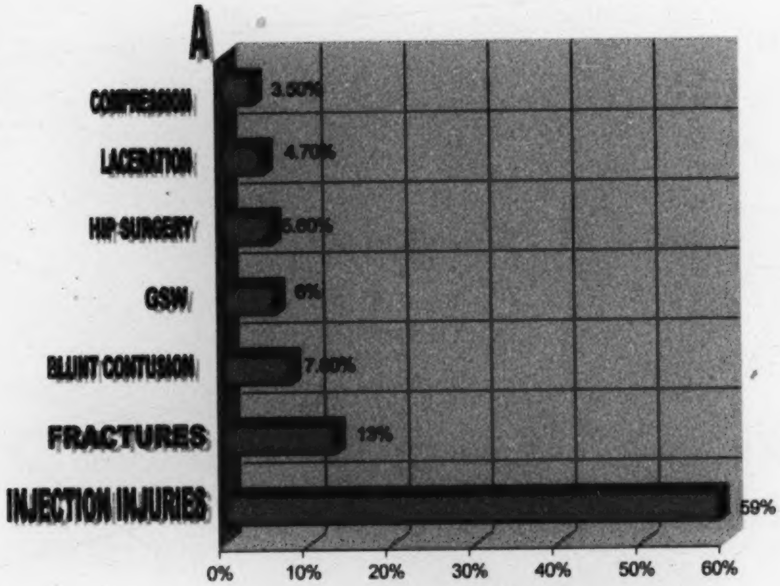


FIGURE 1.3A: CAUSES OF BUTTOCK LEVEL SCIATIC NERVE INJURIES (KLINE ET AL., 1998)

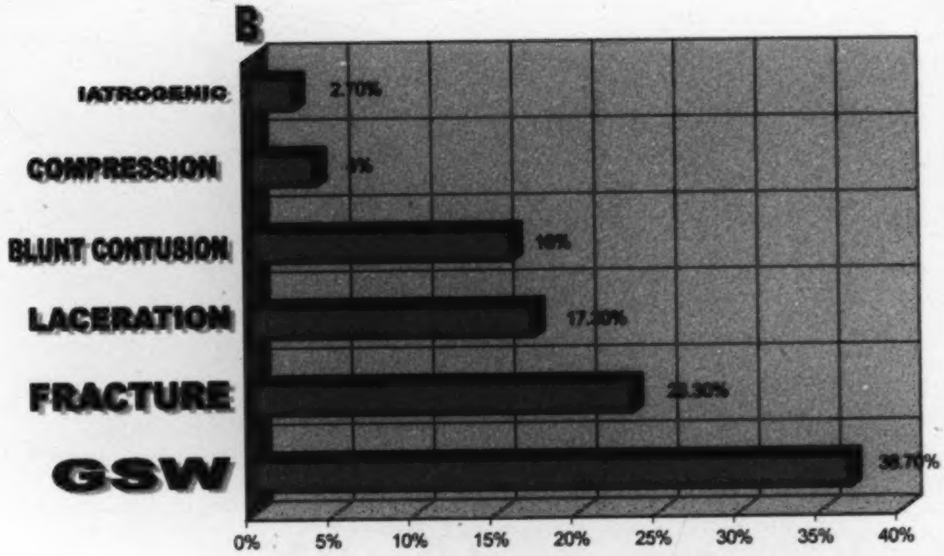


FIGURE 1.3B: CAUSES OF THIGH LEVEL SCIATIC NERVE INJURIES (KLINE ET AL., 1998)

1.2 CELLULAR AND MOLECULAR CHANGES AFTER PERIPHERAL NERVE INJURIES (PATHOGENESIS)

Injury to peripheral nerves results in a multitude of complex but highly reproducible sequence of histopathological events in both the injured neurons and their peripheral nerve projections. There are predictable changes centrally at the cell body, within the proximal nerve stumps, at the site of nerve injury, and within the distal nerve stump, as well as in the end organs of motor end plates or specialised sensory receptors.

1.2.1 CHANGES IN THE INJURED NEURONS

Within hours after nerve injury, an anatomical process of 'chromatolysis' is initiated in the injured neurons which is characterised by rounding of the cell body, with migration of the nucleus to a peripheral location near the axon hillock and nucleolar enlargement and associated with an increase in the RNA content of the cell body (for reviews see Lieberman, 1971, 1974; Kreutzberg, 1995; Fu & Gordon, 1997). Peripheral nerve injuries in adults are followed by significant (20-40%) loss of dorsal root ganglion (DRG) neurons (Schmalbruch, 1987; Himes & Tessler, 1989; Liss et al., 1996; Groves et al., 1997), most probably due to apoptosis (Edstrom et al., 1996; Groves et al., 1997) and, to a much lesser extent, loss of motoneurons, depending on the distance of the injury sites to the neuronal cell bodies. Death of spinal motoneurons in adult mammals is rare as sites of injuries are often far from the cell bodies (Himes & Tessler, 1989; Melville et al., 1989; Gordon et al., 1991) compared to the high rate of cell death of the cranial motoneurons in which injuries occur in close proximity to the cell bodies (e.g. cranial nerve lesions; Arvidsson & Aldskogius, 1982; Snider & Thanedar, 1989).

1.2.1.1 Changes in Gene Expression

One of the earliest neuronal cell body responses to peripheral nerve injuries is the rapid induction and long-term expression of the immediate early gene transcription factors *c*-Jun (Leah et al., 1991; Kenney & Kocsis, 1998). Injured motoneurons progressively lose their expression of choline acetyltransferase (see below), increase their expressions of tropomyosin receptor kinase-B (trkB), the signal transducing receptor for brain-derived neurotrophic factor (BDNF) and neurotrophin-4/5 (NT-4/5), and the neurotrophic receptor, p75NTR (Ernfors et al., 1989; Friedman et. al, 1995; Kobayashi et al., 1996; reviewed by Fu & Gordon, 1997; Terenghi, 1999), although the upregulation of p75NTR has been related to axonal regeneration rather than to a signal of nerve damage (Rende et al., 1993). Motoneuronal expressions of glial-derived neurotrophic factor (GDNF) and its signal transduction component, the tyrosine kinase RET, calcitonin gene-related peptide (CGRP), the fast transported growth-associated protein-43 (GAP-43) and the slowly transported cytoskeletal proteins, actin and tubulin are also upregulated but neurofilaments are downregulated (Haas et al., 1990; Tetzlaff et al., 1988, 1991, 1996; Naveillan et al., 1997). In the injured sensory neurons, several neuropeptides are either upregulated or downregulated, for example, substance P, CGRP, and somatostatin are downregulated after injury whereas vasoactive intestinal protein, galanin, neuropeptide Y and cholecystokinin are upregulated (Verge et al., 1996; reviewed by Zigmond et al., 1997). These changes in the gene expressions after nerve injuries probably reflect the importance of the upregulated/downregulated molecules in the events subsequent to nerve injury such as, survival and axonal regeneration, although direct evidence of the involvement of these molecules are limited.

1.2.1.2 Phenotypic Changes from Impulse Conduction to Growth

Following nerve injury, metabolism in the cell body shifts from production of neurotransmitters to the synthesis of membrane proteins. Hence, the synthesis of cytoskeletal proteins such as actin and tubulin is increased in an effort to reconstruct the axon cytoskeleton whereas synthesis of the cholinergic synthetic rate-limiting enzyme, choline acetyltransferase (CAT) is reduced (for review see Fu & Gordon, 1997).

1.2.2 CHANGES IN THE NERVE STUMPS DISTAL TO THE INJURY SITE

In addition to the pathophysiological and phenotypic changes in the injured neurons, there are certain pathognomonic changes in the injured nerve stumps. In 1850, Waller described these changes in the nerve stumps distal to injured frog glossopharyngeal and hypoglossal nerves. Thus, *Wallerian degeneration* refers to the specific changes in the distal stumps of injured myelinated nerves. However, these changes are not limited to the distal nerve stumps, as they are also seen in the proximal nerve stumps up to or beyond the first node of Ranvier, depending on the severity of the injury and in nerve autografts (for review see Diao & Vannuyen, 2000).

1.2.2.1 The Inflammatory Response: Wallerian Degeneration

The complex cell-molecular interactions that ensue between the SC and macrophages which infiltrate the injury site are essential for the provision of growth-permissive environment for regenerating axons (Chen & Bisby, 1993). Following injury, the axon distal to the injury site is disconnected from the cell body and undergoes Wallerian degeneration (Abercrombie & Johnson, 1946). Wallerian degeneration is characterised by axonal and myelin breakdown induced by the activation of axonal proteases and calcium influx (Schlaepfer & Bunge, 1973). Degraded myelin and axonal debris undergo phagocytosis, initially (first 3 days) by SCs which express complement factor 3 (C3; Liu et al., 1995) and later by both SCs and macrophages recruited to the injury site (Adams & Johnson, 1992; Reichert et al., 1994; Dashiell et al., 1997; for review see Muller & Stoll, 1999; Diao & Vannuyen, 2000). It has been suggested that, in addition to other chemoattractive forces, SCs help to recruit macrophages to the injury site via the secretion of the inflammatory cytokine, interleukin-1β (IL-1β), in the first 3 days after nerve injury (Bergsteinsdottir et al, 1991; Watkins et al., 1995). Other macrophage- and SC-derived cytokines have been implicated in phagocytosis: IL-1β and tumor necrosis factor-α (TNF-α) increase while transforming growth factor-β (TGF-β) reduces the phagocytic activity of SCs and macrophages (Sporn et al., 1987; Tsunawaki et al, 1988; Chandross et al., 1995). Active phagocytosis of myelin debris by macrophages is essential for axonal regeneration because i) myelin-derived proteins (NI-35, NI-250 and Nogo-A) and MAG have been shown to inhibit neurite growth in the CNS and PNS (Chen, 2000; Grandpre et al., 2000; reviewed by Bandtlow & Schwab, 2000), ii) SC migration and proliferation AND axonal regeneration are severely retarded in the C57Bl/Ola mouse mutant where macrophage invasion is sluggish and Wallerian degeneration is delayed (Bisby & Chen, 1990; Brown et al., 1992; Chen & Bisby 1993).

1.2.2.2 Changes in the Schwann Cells

Loss of axonal contact induces a series of changes in the SCs of the distal nerve stumps that range from proliferation to changes in their phenotype. These changes create a microenvironment that supports axonal regeneration by the axotomised neurons either by retrograde transport of secreted growth factors or by expressing surface molecules that are positive substrate for both axonal growth and elongation.

1.2.2.2.1 Proliferation and Changes in Phenotype

Loss of axonal contact initiates SC proliferation and a switch in gene expression in the SCs from the myelinating to the non-myelinating phenotype in which myelin-associated proteins such as protein zero (P_0) myelin basic protein (MBP), MAG and PMP22, are downregulated and p75NTR, glial fibrillar acidic protein (GFAP), and GAP-43 are upregulated (Jessen et al., 1990, 1991; Scherer & Salzer, 1996; Mirsky & Jessen, 1999; Stoll & Muller, 1999). SC proliferation has been associated with their autocrine capability to synthesize and express growth factors including, the neuregulins (neu differentation factor, glial growth factor, heregulin) and their erb receptors B1, B2, and B3 (Raabe et al., 1998; Adlkofer and Lai, 2000), as well as neurotrophic factors such as nerve growth factor (NGF) and BDNF which may have autocrine actions via p75NTR receptor (Eccleston et al., 1989; Ridley et al., 1989; Matsuoka et al., 1991, 1997; Rogister et al., 1993; Bibel et al., 1999). Macrophages also secrete numerous growth factors that are mitogenic for SCs. These include platelet derived growth factor (PDNF), NGF, epithelial growth factor (EGF) and other neurotrophic factors like basic fibroblast growth factor (bFGF) (Davis & Stroobandt, 1990; Higashiyama et al., 1991; Heldin, 1992; Rogister et al., 1993; Matsuoka et al., 1997; Davies, 2000).

The importance of cytokines in peripheral nerve regeneration and in SC-macrophage interaction has been demonstrated in several experiments (Hopkins & Rothwell 1995). Interleukins 1, 6 and 10 are upregulated in the distal nerve stumps after nerve injury (Bolin et al., 1995; Bourde et al., 1996; Reichert et al., 1996; Gillen et al., 1998). IL-1β has been shown to induce NGF in fibroblasts and implicated in maintaining NGF in SCs (Lindholm et al., 1987). Macrophages can upregulate the expression of p75NTR by SCs via the production of PDGF (Lindholm et al., 1987). TGF-β secreted by macrophages and SCs has the same effect as cAMP in the induction of NGF-mRNA in SCs, an effect which is augmented by PDGF and bFGF (Mutsuoka et al, 1991; Meyer et al, 1992; Schubert, 1992). However, effects of TGF-β on SCs are state-dependent as in other cells (Sporn et al, 1987; Einheber et al., 1995). For instance, TGF-β is mitogenic for SCs in the presence of serum in vitro but inhibits proliferation of SCs once they express the non-myelinating phenotype or when they are co-cultured with DRG neurons (Stewart et al., 1991; Guenard et al., 1995b). In vitro TGF-β promoted the non-myelinating growth-supportive phenotype of SCs while blocking their expression of the myelinating phenotype (Guenard et al., 1995a, b).

1.2.2.2.2 Growth-supportive Changes

After the phagocytic removal of axon and myelin debris, proliferating SCs form a strand of cells called the SC column or bands of Bungner (Bungner, 1891) within the basal lamina tube. These bands of Bungner are essential for guiding regenerating axons to their denervated targets (Cajal, 1928). The regenerating axons extend along the surface of SCs and/or the inner surface of the basal lamina of the SC column, which contain a variety of adhesion molecules that regenerating axons could use as substrates for attachment and growth (Ide et al., 1983; Kleitman et al., 1988; for review see Ide, 1996; Weinstein, 1999). Axon-SC attachment and SC myelination of axons are mediated by various adhesion molecules including the immunoglobulin superfamily, e.g. N-CAM and L1, and the cadherin superfamily, e.g. N-cadherin and E-cadherin, and laminin whereas axon-basal lamina contact is, for the most part, mediated mainly by laminin (Bunge &Wood, 1987; Wood et al., 1990; Fernandez-Valle et al., 1994; see reviews by Bixby & Harris, 1991; Letourneau et al., 1994; Chernousov & Carey, 2000). After nerve injury and upon reinnervation, SCs upregulate N-CAM, L1, N-cadherin (Martini and Schachner, 1988; Cifuentes-Diaz et al., 1994) and basement membrane components including laminin (Bunge & Bunge, 1983; Daniloff et al., 1986; Bixby et al., 1988; Brodkey et al., 1993; for review see Chernousov & Carey, 2000). Hence, distal nerve stumps that lack either SCs or basal lamina cannot provide optimal support of axonal regeneration (Hall, 1986a, b; reviewed by Ide et al., 1990; Weinstein, 1999).

An important component of the non-myelinating phenotype of the SCs is the expression of growth factors which include three members of the neurotrophin family, NGF, BDNF, and NT-4/5, and their common receptor, p75NTR, as well as EGF and its receptors, insulin-like growth factors I and II (IGF-I; IGF-II), and GDNF and its GFRα receptor, all of which have been associated with axonal growth (Hanson et al., 1986; Meyer et al., 1992; Toma et al., 1992; Funakoshi et al., 1993; Trupp et al., 1995; Naveilhan et al., 1997; You et al., 1997; Hammarberg et al., 1998; reviewed by Althaus & Richter-Landsberg, 2000). The effects of neurotrophins on motor axonal regeneration is discussed in section 1.3.2.3 (see below).

GDNF has been demonstrated to be an extremely potent survival factor for motoneurons (Henderson et al., 1994). In rodents whose motoneurons die as a consequence of genetic mutations or nerve lesions, administration of GDNF protein, injection of adenoviral vectors harboring a GDNF gene, or implantation of encapsulated cells secreting GDNF have demonstrated protective effects (reviewed by Bohn, 1999; Airaksinen et al., 1999). IGF-II has been shown to support the survival of neonatal motoneurons after sciatic nerve injury (Pu et al., 1999). Further evidence to support the importance of IGFs in motoneuronal survival and regeneration includes the observation that IGF-binding protein-6 was strongly upregulated in spinal motoneurons under different injury models (Hammarberg et al., 1998) and that IGFs increased the expression of GAP-43 by motoneurons in vitro, respectively (Piehl et al., 1998). The exact effects of EGF on motoneurons remain to be demonstrated.

1.3 THERAPEUTIC APPROACHES

The main therapeutic option after peripheral nerve injuries remains surgical despite several important neurobiological observations and better understanding of the pathophysiology of nerve injury. Many of the significant experimental results are yet to find clinical applicability after nerve injuries (Mckinnon & Dellon, 1988; Gielberman, 1991; Kline, 2000).

1.3.1 Microsurgical Repair of Injured Nerves

Significant advancements have been made since World War II in surgical repair of severed nerves, especially in the fields of microsurgical techniques for nerve repair, nerve grafting, and intraoperative neurophysiological evaluation of nerve lesions and regeneration (Slimp, 2000; Kline, 2000). The invention of operating microscope, loupes and development of microinstruments, such as microdissectors, microscissors and fine forceps provide sufficient magnification and enhance the precision of nerve repair tremendously. Another significant development was the bipolar coagulation, that permits hemostasis of bleeding points at the epineurial and interfascicular neural levels, with minimal associated damage to the nerve itself. Therefore, it is understandable why many believe that advancements in microsurgical techniques to improve nerve repair and regeneration have been exhausted and, that any further advancements would have to be biochemical in nature, which could further aid regeneration and/or to modulate the inhibitory environment of the scar tissue (Fu & Gordon, 1997; Hudson et al., 2000).

1.3.1.1 Strategy and Timing of Peripheral Nerve Microsurgery

While it is generally accepted that the clinical treatment of a severed nerve is microsurgical repair, the strategy adopted by surgeons differ and the timing of nerve repair remains controversial. However, more clinical studies propose that most nerve injuries must be considered true surgical emergencies and should be operated on within hours of nerve injury, i.e. primary repair should be performed as often as possible (Bunnel, 1956; Kline et al., 1974; van Beek et al., 1975; de Medinaceli et al.,, 1983; Merle and de Medinaceli, 1992). Although the necessity for a primary nerve repair was questioned by Millesi et al., (1972) with the tremendous improvement in fascicular nerve grafting technique, a recent review by Millesi (2000) proposes the following: i) if there is a clean transection and the distance between the two stumps is caused by elastic retraction, an end-to-end neurorrhaphy has a very good chance of success, ii) if there is a defect caused by loss of nerve tissue, the chances of success decrease in inverse proportion to the length of the defect, and a graft procedure in the form of a secondary repair is preferable.

1.3.1.1.1 Nerve Repair Technique

Detailed illustrations of the techniques of peripheral nerve repair have been described previously (Bowers et al., 1989; Merle & de Medinaceli, 1992; Diao & Vannuyen, 2000; Matsuyama et al., 2000; Spinner & Kline, 2000). The goal of any repair technique is to coapt the severed nerve accurately without any tension and if possible, in the proper rotational alignment, using the longitudinal blood vessels on the nerve as a landmarks during alignment. Nerve repair techniques include epineurial, group fascicular, fascicular and mixed repairs, all of which should be performed under operative magnification, using either magnifying loupes or the operating microscope, and nonabsorbable 7-0, 8-0, 9-0, or 10-0 sutures, depending on the caliber of the nerves. The first step prior to nerve repair is debridement, during which damaged nerve ends are dissected until viable nerve ends are visible. Palpation of the ends of the nerve with a microforceps often allows the surgeon to determine a region of swelling or thickening of the nerve. The choice of nerve repair technique is dependent on the size of the nerve being repaired, with the primary objective of optimising the functional outcome following the repair. Thus, epineurial repair is used for repair of nerve with very few fascicles and, fascicular and/or group fascicular repair is used for the repair of large nerve with multiple fascicles.

1.3.1.1.2 Primary Versus Secondary Repair

Nerve repair performed within the first week of injury is termed primary, although it is considered 'delayed primary repair' when performed after first 3 to 4 days after injury (Diao & Vannuyen, 2000). However desirable it is, primary nerve repair can be performed only under the following conditions: i) a sharp nerve transection with a limited zone of injury within a clean wound environment; ii) minimal or no associated bone, joint, or soft tissue injuries, along with circulatory, metabolic, and psychiatry stability of the patient; and iii) appropriate microsurgical instrumentation and personnel trained in the techniques of microsurgery, especially possessing the skills to accurately coapt severed nerve endings without undue tension.

When these conditions are not met and nerve repairs are performed more than one week after nerve injury, they are said to be secondarily repaired (Bowers et al., 1989; Merle & de Medinaceli, 1992; Diao & Vannuyen, 2000). Secondary repair is often warranted in patients with war wounds; the time between the injury and repair allows for decontamination or debridement of the wounds. When the defect between nerve endings is too large, nerve grafting procedures should be performed to minimise tension across the suture, and thereby reduce scaring (Millesi, 2000).

1.3.1.2 POOR FUNCTIONAL RECOVERY AFTER NERVE REPAIR

Functional recovery is often delayed and unsatisfactory after nerve injuries (Fu & Gordon, 1997; Allan, 2000). In an extensive review of functional recovery in 231 patients throughout the 24 years after sciatic nerve injury and microsurgical repair, Kline et al (1998) found that the average period before some recovery was evident was10-14months for tibial division and more than 16 months for peroneal division injuries. The distance which axons of the injured neurons have to traverse before reaching their targets also influenced functional recovery, as sciatic injuries at the thigh levels recovered better compared to injuries at the buttock levels. Recovery of useful function was greatest after neurolysis if associated with a positive nerve action potential, intermediate after suture, and less likely but possible after graft repair. The severity of the initial injuries probably reflected on the outcomes of the different therapeutic approaches. Overall results were poor for resection and suture or graft repair for peroneal compared with tibial nerve divisions, except in children.

Brachial plexus injuries exhibit similar patterns of functional recovery. Approximately 70% of patients that had undergone microsurgical reconstruction with nerve grafting and/or neurotisation of the injured brachial plexus did not notice improved movement and sensibility until after several months and only 50% reached maximum function after 4 years (Choi et al., 1997). Functional recovery/reinnervation was more delayed in the more distal targets, as 78% of patients studied noted full or partial movement proximally in both their shoulder and elbow, while only 63% experienced full or partial return of both their wrist and finger movements. Likewise, functional recovery after brachial plexus injuries was better after neurolysis than grafting (Kline et al., 1983, 1986; Millesi, 1984, 1987; Millesi, 2000).

1.3.2 EXPERIMENTAL APPROACHES TO IMPROVE NERVE REGENERATION AND FUNCTIONAL RECOVERY

Since it is unlikely that improved clinical results (i.e., recovery of muscle and sensory function) will come from further progress in microsurgical procedures, several experimental approaches which are designed based on current understanding of neurobiology of nerve regeneration, are now being explored to determine their clinical applicability to improve nerve regeneration after injuries in humans. Below, I discuss some of the recent advances in nerve repair techniques and some of the experimental approaches to augment nerve regeneration after nerve repair.

1.3.2.1 Nerve Grafting Techniques

The clinical treatment of a severed peripheral nerve involves either surgical realignment of the injured nerve stumps (i.e. primary neurorrhaphy) or the use of an autologous nerve graft to bridge a larger defect. Phillipeaux and Vulpian, Gluck, Albert, and Mayo-Robson were the first to report on the technique of nerve grafting during the years 1870 through 1900 (reviewed in Walker, 1951). They found that a nerve restoration by grafting yielded a superior result compared with direct repair under tension. However, it was Millesi (1972, 1976, 1981, reviewed in 2000) who wrote extensively on the subject of nerve grafting. His work demonstrated that i) nerve grafting without tension was superior to epineurial suture under tension, ii) tension at the repair site induces scar formation and hence, nerve repair without tension is most desirable, because the more scar tissue present at the repair site, the less satisfactory functional recovery. Tension across a direct suture repair decreases blood flow (Miyamoto, 1979) and promotes proliferation of connective tissue within the nerve, which may block effective axonal regeneration (Rodkey et al., 1980). Acute, excessive stretch may cause intraneuronal hemorrhage, resulting in scar formation and axonoplasmic degeneration; subsequent maturation of scar tissue may shrink and constrict the nerve fibers and, may result in the formation of a neuroma in continuity (Matsuyama et al., 2000).

The use of nerve grafting technique when the gap between the two nerve stumps is small-to-moderate is controversial (Matsuyama et al., 2000). In nerve grafting, the regenerating axon sprouts must cross two suture sites of coaptation. While Millesi (2000) argues that if tension at the sites of coaptation is completely avoided, the axons sprouts are able to cross two sites of repair more easily than one site under unfavourable conditions, others believe that scar tissue at the distal site of coaptation may block the crossing of axons from the graft to the distal nerve stump (Bratton, 1979) and, axons may be wasted at both the proximal and distal suture lines by being misdirected into the perifascicular and epineurial connective tissue (Hudson et al., 1979; reviewed by Matsuyama et al., 2000). Therefore, it is advocated that regeneration through mildly stretched direct nerve repair and functional recovery thereafter, are of equal value to meticulously executed graft repair (Bratton, 1979; Hudson et al., 1979). It was found that the effect of two suture lines is more deleterious to axonal regeneration than the tension created by direct suture of severed nerve with small gap, although there is a minor decrease in regeneration due to the degree of tension (Wong & Scott, 1991).

Nerve grafting techniques include the use of either *'free nerve graft'* which could be in the forms of cable graft, interfascicular nerve graft, split nerve grafts/end-to-side coaptation OR of *'vascularised nerve graft'* in the form of pedicle grafts or as a free vascularised graft transfer (Strange, 1947; Taylor & Ham, 1976) . The use of vascularised nerve graft is said to minimise the reliance of the grafts on adhesion ingrowth for revascularisation and as such, they can be used in poorly vascularised areas and find major application in brachial plexus surgery (reviewed by Millesi, 2000).

1.3.2.1.1 Nerve Tissue Grafts

Nerve grafts can be classified according to their biologic origin: *Autografts* are harvested from the same individual; *Allografts* (or homografts) come from an individual of the same species, and *Xenografts*, (or heterografts) are from an individual of another species.

1.3.2.1.1.1 Autografts

Autografts, which are the gold standard for nerve grafting surgery, are harvested from the same patient and do not pose immunologic problems. The use of nerve trunks as grafts is no longer practised since they were too thick to be revascularised quickly enough to avoid central necrosis and due to unacceptable donor site morbidity unless harvested from a nonreplantable amputation (Wyrick & Stern, 1992). Nowadays, the donor nerve used for nerve grafting are commonly expendable sensory nerves which can be harvested without causing major problems, except some loss of sensibility at the donor sites. Commonly used donor nerves include the sural nerve, lateral antebrachial cutaneous nerve, anterior division of the medial antebrachial cutaneous nerve, dorsal cutaneous branch of the ulnar nerve, and superficial sensory branch of the radial nerve (Matsuyama et al., 2000). The choice of donor nerve to be used is dictated by the cross-sectional area of the nerve to be repaired, the length of the nerve gap and the extent of the donor site morbidity. The main donor nerve for free grafting is the sural nerve, which can be excised to a length of 30 to 50 cm and is easily assessible (Matsuyama et al., 2000; Millesi, 2000).

1.3.2.1.1.2 Allografts (Homografts)

The use of nerve grafts obtained from another human being is warranted in situations where, i) repair of large defects created by some brachial plexus or sciatic nerve injuries is limited by the inadequate number of available segments from autografts, ii) harvesting of nerves from the same patient is undesirable because of the need for additional surgery and the possibility of causing sequelae such as painful neuroma at the donor site. However, since allografts trigger immunologic response and there is a risk of rejection, the use of immunosuppressive treatment is required to prevent rejection. The need for immunosuppressive therapy gradually wanes as the allogeneic SCs are replaced by autologous

SCs, and the therapy is eventually stopped (Millesi, 2000). Although the use of allografts is being studied extensively in animal models (Mckinnon et al., 1985, 1992; Midha et al., 1993, 1997, 1998, 2001), only partial success has been reported with the use of allografts to repair a defect of the sciatic nerve in a human patient(Mckinnon & Hudson, 1992; Mckinnon et al., 1997). An experimental technique of combining immunosuppression with injection of cultured host SCs into nerve allografts has been shown to improve axonal regeneration into the allografts (Ogden et al., 2000). This technique, combined with recent advancements in the harvesting and in vitro expansion of human SCs (Casella et al., 1996), may increase the success rate of clinical use of allografts.

1.3.2.1.2.3 Xenographs (Heterografts)

Xenografts are derived from individual of another species and they are also used in similar situations as outlined above for allografts and require the use of immunosuppressive therapy. The use of xenografts is thus far, limited to experimentations in animals.

1.3.2.1.2 Artificial Grafts (Guidance Channels)

Donor site morbidity can be a nuisance in autologous nerve grafting, especially when additional and extensive surgery is required to harvest the grafts, and the use of allo- or xenografts is still mostly experimental. In addition, functional recovery after both direct nerve repair and secondary repair with or without grafts, is still suboptimal. Hence, there is the need for alternative strategies to optimise nerve regeneration and eliminate the use of nerve grafts and the adverse effects associated with their use.

Recent advances in neurosciences, especially in the field of neurobiology of nerve growth and regeneration (*in vivo* and *in vitro* observations), genetic engineering and the development of novel biomaterials provide insights into the development of new therapeutic approaches to improve surgical treatment of peripheral nerve injuries. Natural or synthetic guidance channels are being developed as alternatives to nerve autografts. Guidance channels help direct axonal sprouts from the proximal stumps to the distal nerve stumps, they also provide a conduit for diffusion of neurotropic and neurotrophic factors secreted by the SCs of the injured distal nerve stump, and minimise infiltration of fibrous tissue (Hudson et al., 2000). They may also be used as means of delivery of molecules such as extracellular matrix molecules (e.g. laminin, fibronectin, and some forms of collagen) and growth factors (e.g. NGF, BDNF, IGF-1 and IGF-II, PDGF, bFGF and aFGF, CNTF) that have been shown to promote nerve regeneration (Fu and Gordon, 1997; Hudson et al., 2000).

Hudson et al., (2000) described the properties of nerve conduits that have potentials for clinical applicability as follows, i) guidance channels with porous or biodegradable channel wall or both, to allow diffusion of small growth-enhancing factors or degradation of the channel over time; ii) channels which can release soluble neurotrophic factors either by direct incorporation of the molecules or by controlled release from the channel wall, iii) channels transplanted with support cells such as SCs that secrete growth factors and provide a matrix for axonal elongation or other cells that have been genetically engineered to secrete neurotrophic or survival factors, iv) channels that have highly porous and aligned intraluminal matrix to support cell attachment and migration and v) channels with smaller intraluminal channels to aid with fascicular organisation of the regenerating nerve, and vi) channels with inherent electrical properties (Figure 4). Guidance channels with all of these properties will be an ideal channel and will be superior to autografts, although clinical use of guidance channels is still very limited. Synthetic (Polyglycolic acid, PGA and expanded polytetrafluoroethylene, ePTFE) and natural (vein) conduits have been used clinically to repair short gaps (range of 1-4cm) in injured nerves with excellent sensory recovery but limited motor recovery (Mckinnon & Dellon, 1990; Chiu & Strauch, 1990). Incorporation of the aforementioned properties of an ideal channels into the guidance channels are being actively explored experimentally. Use of nerve conduits seeded with SCs have been shown to be superior to autografts (Hadlock et al., 2000) and a combination of SC-seeded nerve conduits and application of glial growth factor was found to improve both the myelination and conduction velocity of regenerated nerves (Bryan et al., 2000).

Therefore, systematic improvements in the properties of guidance channels that are currently being used in the repair of injured human nerves, which include integration of properties of guidance channels that have been shown to exert positive effects experimentally, may further promote functional recovery and allow repair of larger defects.

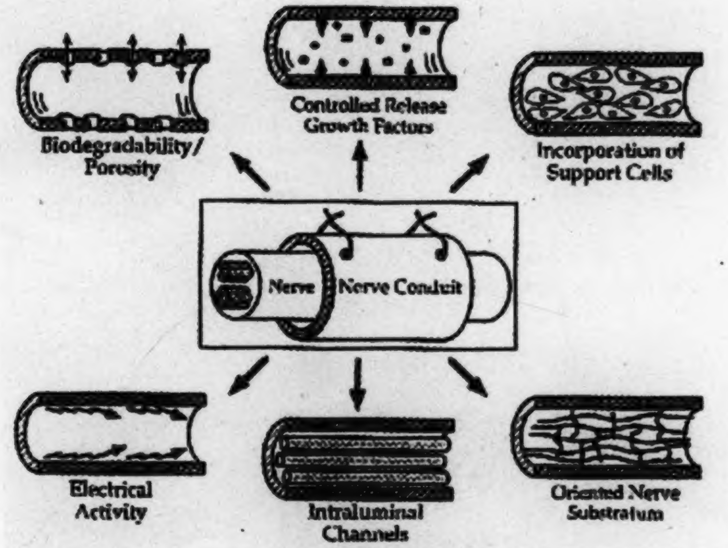

Biodegradability/
Porosity
Controlled Release
Growth Factors
Incorporation of
Support Cells
Nerve
Nerve Conduit
Electrical
Activity
Intraluminal
Channels
Oriented Nerve
Substratum

Figure 1.4: Components of the ideal guidance channel (from Hudson et al., 2000)

1.3.2.3 Functional Electrical Stimulation (FES)

Application ofFES to promote axonal regeneration after peripheral nerve injuries was triggered by separate reports that electrical stimulation accelerated both the onset of sprouting from intact axons after partial denervation, and the initiation of electromyographic signals and functional recovery after nerve crush (Hoffman, 1952; Maehlen & Nja, 1982; Doucette & Diamond, 1987; Nix & Kopf, 1983; Pocket & Gavin, 1985; Manivannan & Terakawa, 1994). The effects of electrical stimulation on axonal regeneration after nerve transection were extensively studied recently by Al-Majed et al., (2000). They demonstrated, in a rat model of nerve injury and repair (femoral nerve) that, 1hr electrical stimulation accelerated motor axonal regeneration and promoted preferential motor reinnervation by inducing accelerated upregulations of mRNAs for BDNF and trkB, Tα1-tubulin and GAP-43 in motoneurons (Al-Majed et al., 2001a,b). Therefore, functional elecrical stimulation is a potential tool for promoting functional recovery after nerve injuries, although further experiments are warranted to examine its efficacy in promoting nerve regeneration after secondary nerve repair with grafts, and after injuries to large nerve trunks, and its exact mechanisms of action remain to be fully elucidated.

1.3.2.3 Neurotrophins

Neurotrophins are a family of structurally related proteins that play an essential role in the development, maintenance and regulation of neuronal function in both the central and peripheral nervous systems (Davies, 2000). The identified and somewhat characterised neurotrophins include nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3), neurotrophin-4/5 (NT-4/5) and neurotrophin-6 (NT-6).

The distal stump of injured nerve exerts tropic and trophic influences on regenerating axons (Cajal, 1928). The SCs of the distal nerve stumps produce neurotrophins such as NGF, BDNF and NT-4/5 after nerve injury and, regenerating axons have shown to extend along the density gradient of neurotrophins into the distal nerve segment (Kuffler, 1986; Ide, 1996; for review see, Fu & Gordon, 1997; Althaus & Richter-landsberg, 2000). Neurotrophins also regulate dendritic and axonal morphology *in vivo* (McAllister et al., 1999; Gallo & Letourneau, 2000). Therefore, SCs and the growth factors they produce are essential for nerve regeneration after injuries. However, despite the production of these neurotrophins by SCs and optimised microsurgical nerve repair, abnormalities of function often persist, suggesting that production of neurotrophins by SCs might be suboptimal. Furthermore, clinical evidence showed that NGF levels are reduced, instead of increased, in injured human nerve stumps biopsied during brachial plexus surgery (Birch, 1993). Therefore, it seems that, in addition to reapproximation of the transected nerve ends, adjunct management of nerve injuries in the future may need to include exogenous administration of selected growth factors at adequate concentrations to enhance nerve regeneration. Outlined below are some of the experimental data on the neurotrophins to support their potential capacity to promote peripheral nerve regeneration after injuries and probably improve functional recovery. Since recovery of motor function is more unfavourable than sensory in the clinical scenarios, and experiments in this thesis examined only motor regeneration, the potential applicability of the neurotrophins to augment motor axonal regeneration is emphasised.

1.3.2.3.1 Nerve Growth Factor (NGF)

NGF is the prototype neurotrophin and it has been well-characterised since its purification from male mouse salivary glands (Levi-Montalcini & Hamburger, 1953). It binds to two distinct receptors, a high-affinity, tyrosine kinase receptor (trkA) and the p75NTR receptor which also binds other neurotrophins. Survival of embryonic dorsal ganglion neurons, subsets of sensory and sympathetic neurons is supported by NGF during critical stage of development (Deckwerth & Johnson, 1993; reviewed by Yin et al., 1998). Likewise, NGF regulation of the differential expression of neuropeptides by subsets of trkA-expressing DRG sensory neurons has been linked to its capacity to ameliorate degenerative changes in this subset of neurons after nerve injury (Lindsay and Harmar, 1989; Verge et al., 1996; reviewed by Terenghi, 1999). *In vitro*, exogenous application of NGF enhanced neurite outgrowth of embryonic and adult DRG sensory neurons (Whitworth et al., 1995, 1996; Lindsay, 1988).

Evidence that NGF level is reduced after nerve injury in human suggests that exogenous application of NGF may enhance nerve regeneration by providing NGF at optimal concentration. However, while such application may influence trkA-expressing DRG neurons, no effect on motor axonal regeneration is expected as motoneurons do not express the NGF receptor, trkA (Henderson et al., 1993). In fact, it has been shown that NGF has little or no effect on the survival and neurite outgrowth of motoneurons (Arakawa et al., 1990; Henderson et al., 1993; Baraun et al., 1996).

1.3.2.3.2 Brain-derived Neurotrophic Factor (BDNF)

BDNF has 54% homology with NGF and also rescues a subset of sensory neurons from naturally occurring death. It also promotes the survival and outgrowth of both sensory and sympathetic neurons (Sendtner et al., 1992). However, unlike NGF, BDNF also, i) rescues developing motoneurons from naturally occurring death, ii) promotes survival of rat motoneurons *in vivo* (Koliatsos et al., 1993; Yan et al., 1992, 1994) and *in vitro* (Henderson et al., 1993), iii) prevents the cell death of axotomised motoneurons in anterior spinal horns and facial nucleus (Sendtner et al., 1992, 1994; Yan et al., 1992, 1994; reviewed by Yin et al., 1998). These effects of BDNF are mediated by binding to its tyrosine kinase receptor, trk B, a process which is coupled to autophosphorylation of the trkB receptor and a subsequent phosphorylation of target proteins that mediate the different effects of BDNF (For reviews see Lewin & Barde, 1996; Yin et al., 1998; Terenghi, 1999).

The effects of BDNF on motoneurons suggest the that it may be used to promote motor axonal regeneration. Evidence to support a probable role of BDNF in nerve regeneration include the fact that, after nerve injury, axotomised motoneurons upregulate BDNF and its receptors, trkB and p75NTR (Ernfors et al., 1989; Meyer et al., 1992; Funakoshi et al., 1993; Kobayashi et al., 1996; Friedman & Greene, 1999) and, the mRNA level of BDNF is increased several folds in the SCs of the distal nerve stumps (Meyer et al., 1992; for review, see Ide, 1996). Furthermore, BDNF reduce motoneuron death after axotomy in neonatal (Yan et al., 1992) and adult (Novokov et al., 1995; Novikova et al., 1997) animals and when applied exogenously to chronically axotomised motoneurons, it reversed the detrimental effect of chronic axotomy by improving motor axonal regeneration (Boyd & Gordon, 2000). Future application of BDNF to promote nerve regeneration after injuries would need to be properly titrated as some of its effects have been shown to be dose-dependent (Vejsada et al., 1994, 1995; Boyd & Gordon, 2000).

1.3.2.3.3 Neurotrophin-3 (NT-3)

NT-3 is the third neurotrophin to be identified and shares 55% homology with NGF. It binds to the tyrosine kinase receptor C and, supports the survival and differentiation of sensory and parasympathetic neurons (Henderson et al., 1993). NT-3 strongly supports the survival of motoneurons *in vitro* (Henderson et al., 1993), regulates the function of the developing neuromuscular synapses (Lohof et al., 1993), restores denervated muscle mass when applied exogenously (Sterne et al., 1997), and rescues motoneurons from naturally occurring cell death (Yin et al., 1994; reviewed by Yin et al., 1998). Thus, NT-3 may be able to promote peripheral nerve regeneration after injuries but further characterisation of its effects on axonal regeneration after nerve injury is required.

1.3.2.3.4 Neurotrophin-4/5 (NT-4/5)

This is the next neurotrophin to be isolated and it is referred to as either neurotrophin 4 or 5. It has 48% sequence identity to NGF but binds to tyrosine kinase receptor B. NT-4/5 is an extremely potent survival factor for motoneurons (Henderson et al., 1993), attenuates the loss of choline acetyltransferase expression in axotomised adult rat motoneurons (Friedman et al., 1995), and increases the ability of motoneurons to innervate skeletal muscle fibers *in vitro* (Braun et al., 1996). Therefore, NT-4/5 is a potential neurotrophin that can be used to promote motor axonal regeneration after nerve injuries but its specific effects on axonal regeneration after injury need to be further elucidated.

1.3.2.3.5 Neurotrophin-6 (NT-6)

NT-6 is the last to be described of the common neurotrophins and its effects are still being investigated. NT-6 shows 56% homology with NGF and also supports the survival of sensory and sympathetic neurons although it is not as potent as NGF (Gotz et al., 1994). Its effects on motoneurons remain to be clarified.

1.4 OBJECTIVES AND HYPOTHESES OF THE PhD STUDIES

In the experiments in this PhD thesis, using a rat model of nerve injury and repair, my OBJECTIVES were, to understand some of the factors leading to poor functional recovery after peripheral nerve injuries despite the intrinsic capacity of the peripheral nervous system to regenerate axons AND to design experimental strategies that could be applied to counteract the identified factors. Specifically, we hypothesised that, 1) delayed reinnervation (chronic denervation) of the SCs of the distal stumps of injured nerves, due to the slow rate of regeneration of injured neurons and/or delayed nerve repair leads to, i) a progressive deterioration of the growth-supportive environment and ii) reduced responsiveness of the SCs to regenerating axons, which consequently accounts for poor axonal regeneration and functional recovery; 2) neurotisation of the SCs in the endoneurial tubes by a sensory nerve at the same time as a motor nerve regenerate in the same tubes, will reverse the detrimental effects of delayed reinnervation and maintain the growth-permissive environment of the distal nerve stumps for optimal motor axonal regeneration;

3) deterioration of the growth-supportive phenotype of the SCs after chronic denervation is as a result of loss of their active interactions with macrophages which infiltrate the injured nerve stumps and exposure of SCs to some of the factors that mediate these interactions, such as cytokines, may help resuscitate the growth-supportive phenotype and, 4) molecules which accelerate axonal regeneration, such as FK506, may counteract the detrimental effects of the slow rate of regeneration of injured neurons, by preventing both chronic axotomy of the neurons themselves and chronic denervation of the SCs.

Hypotheses 1 and 2 were tested by experiments described in chapters 1 to 3 of this thesis while hypotheses 3 and 4 were the subjects of chapters 4 and 5. Out of the 4 hypotheses, only hypothesis 2 was disapproved. The results of these experiments were quite intriguing and shed more light on why peripheral nerve regeneration is incomplete and the possibility to design new therapeutic strategies to optimise nerve regeneration after peripheral nerve injuries.

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CHAPTER 2

2.0 EFFECTS OF SHORT- AND LONG-TERM SCHWANN CELL DENERVATION ON PERIPHERAL NERVE REGENERATION, MYELINATION AND SIZE

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2.1 INTRODUCTION

The capacity of injured peripheral nerves to regenerate contrasts the inability of their central nervous system counterparts (Ramon y Cajal, 1928; Kiernan, 1979). This regenerative ability in the peripheral nervous system has been attributed to the growth-permissive environment provided by the Schwann cells of the distal nerve stumps (David & Aguayo,1981). However, despite this regenerative capacity, functional recovery after peripheral nerve injury is often disappointing even after immediate microsurgical nerve repair. Clinical experience has established that functional recovery is particularly poor for injuries which sever large nerves such as lumbar plexus nerve trunks (Sunderland, 1978; MacKinnon, 1989; Millesi, 1990; Terzis & Smith, 1990; Fu & Gordon, 1997; Gordon & Fu 1997;). In these cases, regenerating axons must traverse long distances to reinnervate denervated targets. The slow rate of regeneration at 1-3mm/day, translates into months or even years before regenerating axons might be expected to reach denervated targets. The general conception is that regenerating axons eventually regenerate to the targets but it is the inability of these targets to accept reinnervation that accounts for poor functional recovery (Gutmann and Young, 1944; Gutmann 1948; Sunderland 1978;Terzis and Smith 1990). However, it is possible that these slowly regenerating axons cannot even reach muscle targets because they cannot grow through the Schwann cell environment of the distal nerve stumps which has become less trophic or even atrophic after chronic denervation (Sunderland and Bradley, 1950a, b).

Previous experiments in our laboratory demonstrated that chronic denervation of the distal nerve stump and muscle target reduced the number of motor units in chronically denervated muscles reinnervated by freshly axotomised motoneurons (see Fu and Gordon, 1995 b). The reduced number of reinnervated motor units was interpreted to reflect a reduced capacity of the motor axons to regenerate in the chronically denervated nerve stumps as opposed to an inability of chronically denervated atrophic muscle fibers to accept reinnervation. However, the relative roles of the distal nerve stump and denervated muscle in reducing the number of reinnervated motor units after chronic denervation could not be differentiated by measurement of reinnervated motor unit number. It is therefore important to directly determine how many motoneurons regenerate axons into the distal nerve stump after delayed nerve repair. The present study focuses on i) the isolated influence of the chronically denervated distal nerve stumps on the numbers of, motoneurons which regenerate axons and, regenerated axons, ii) the role of muscle denervation atrophy in poor motor recovery, and iii) the interaction of chronically denervated Schwann cells with regenerated axons. Using the same surgical paradigm of cross-suture of freshly axotomised and chronically denervated nerve stumps, we used fluorescent dyes to backlabel and enumerate motoneurons which regenerate their axons into the chronically denervated nerve stumps, counted the number of regenerated axons and determined their size and myelin thickness. Reinnervated muscle mass was also measured. We show that i) after long-term chronic denervation, many motoneurons failed to regenerate their axons into the distal nerve stump, ii) denervated muscle target can accept reinnervation and recover from denervation atrophy, iii) the few regenerated axons that regenerated after long-term chronic denervation were well myelinated by the chronically denervated Schwann cells. These results have been presented in abstract form (Sulaiman et al.,1998).

2.2 MATERIALS AND METHODS

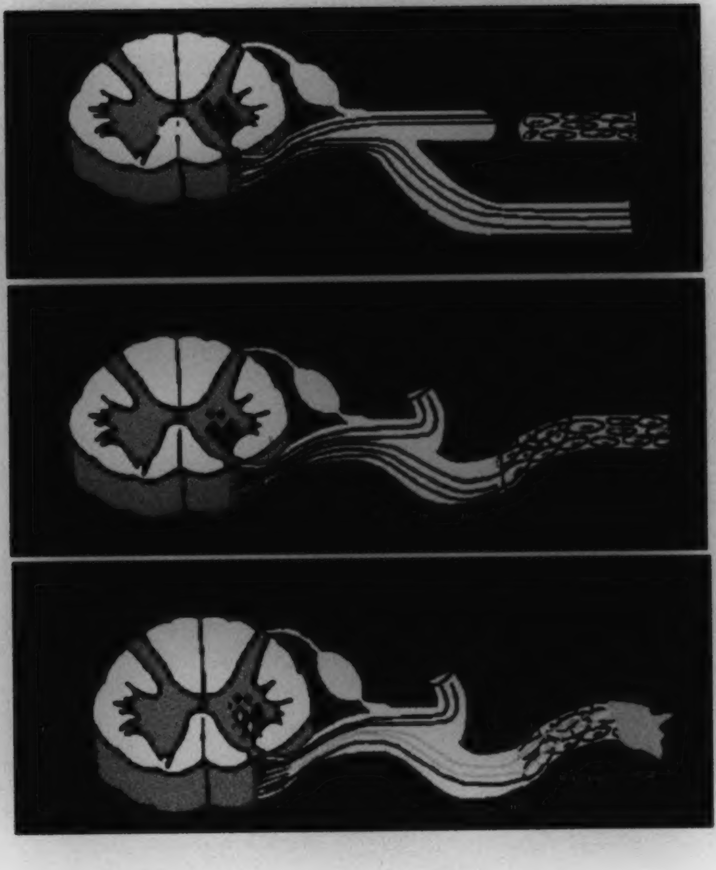
The relative contribution of chronic denervation to poor functional recovery was studied independently by means of a nerve cross-anastomosis paradigm: the common peroneal (CP) nerve was sectioned (so that the target muscle and the distal nerve stump were both denervated) and later cross-suture of the freshly cut foreign tibial (TIB) nerve and the denervated stump was performed (Holmes and Young, 1942; Gutmann and Young, 1944; Fu and Gordon, 1995).

2.1.1 Surgical Procedures

The section and cross-anastomosis of the two branches of the right sciatic nerve, CP and TIB, were used as a model of traumatic nerve injury to study TIB nerve regeneration after chronic denervation of the CP distal nerve stump and the target muscle (Figure1). Female Sprague-Dawley rats were used for all experiments and surgeries were performed under aseptic conditions and deep anaesthesia induced by sodium phenobarbital (30mg/kg i.p.). The right CP nerves of 24 adult female Sprague-Dawley rats were cut and regeneration prevented by ligating both proximal and distal stumps to adjacent innervated muscles (Figure 1A). This way, regeneration of axons from the proximal stump into the distal nerve stump was prevented so that the target muscle and the distal nerve sheath were both denervated. Denervation of the distal nerve sheath was prolonged for periods of 2 days, 1 week, 4 weeks, 8 weeks, 12 weeks and 24 weeks, with 4 rats in each group.

At the end of each denervation period, cross-suture of the proximal stump of freshly

Figure 2.1: Schematic diagram of the nerve cross-anastomosis model to examine the effect of distal nerve stump chronic denervation on axonal regeneration. The CP and TIB branches of rat sciatic nerve were used in this experiment. The CP nerve was cut, and the proximal and distal stumps were separated and ligated to two different innervated surrounding muscles to subject the distal stump to chronic denervation (A). Two days, 1, 4, 12 and 24 weeks later, the TIB nerve was cut and its proximal stump was sutured to the chronically denervated distal CP stump to encourage regeneration of freshly axotomised TIB motoneurons into the chronically denervated CP nerve stumps (B). Twelve months later, axotomised TIB motoneurons which regenerated their axons into the chronically denervated distal CP stump were backlabelled by exposing the axons to neuroanatomical tracers (fluorogold or fluororuby) (C) (see methods)



cut ipsilateral TIB nerve and the distal denervated stump of CP was performed (Figure 1B). This was to facilitate regeneration of the axons of freshly axotomised and regenerating TIB motoneurons through the chronically denervated distal nerve sheaths. Animals were allowed to recover and kept for a period of 12 months to allow TIB motoneurons to regenerate through the denervated stumps and reinnervate denervated flexor musculature. A 12 month period was chosen to allow for recovery of regenerated nerve fibres and reinnervated muscle.

2.1.2 Retrograde Labeling of Motoneurons

In order to determine the number of TIB motoneurons that regenerated their axons through the chronically denervated distal CP nerve stump at the end of 12 month regeneration period, retrogradely transported axonal tracers were used to backlabel TIB motoneurons (Figure1C). Fluorogold (FG; Fluorochrome Inc. Denver) and Fluororuby (RF; Dextran tetramethylrhodamine, Mol. Probes, D-1817, Eugene, OR) were the 2 dyes chosen since they are effectively endocytosed and retrogradely transported (Richmond et al., 1994; Novikova et al., 1997). The CP nerve of each rat was cut 25mm distal to the site of CP-TIB cross-suture for exposure to either FG or FR for retrograde labelling of TIB motoneurons which regenerate their axons into the chronically denervated CP distal nerve stump. A 3mm long piece of reinnervated CP nerve was dissected and excised distal to the site of section and dye application, just before the application of retrograde dyes, for light and electron microscopy. The intact TIB nerve on the contralateral side was also cut and a 3mm long piece of the intact TIB nerve was removed at the same level for comparison. The choice of dye was alternated between animals to control for possible differences in retrograde uptake and transport of the dyes. In addition, the contralateral freshly cut TIB nerve was labelled with either FG or FR, after the removal of the 3mm piece of nerve, in order be able to enumerate the total number of motoneurons that normally send axons down the TIB nerve. Backlabeling with FG was done by exposing the tip of the cut CP nerve to 4% FG in 0.1 M cacodylic acid for 1 h (Schmued and Heimer, 1990) in a vaseline well, after which it was extensively irrigated and reflected to a distant portion of the wound. Backlabeling with FR was done by placing the tip of the cut CP nerve on a 1cm^2 weighing paper (Fisher Scientific Co., USA) with FR crystals for 2 h, then irrigating the nerve and placing it in the opposite corner of the wound to prevent backlabeling of non-TIB motoneurons by diffusion of tracers. Animals were allowed to recover and kept for 3 days to allow for retrograde transport of the applied fluorescent dyes to the neuronal cell bodies.

2.1.3 Tissue Perfusion and Nerve Histology

On the 3rd day after the application of the retrograde dyes, rats were again deeply anaesthetized with sodium phenobarbital (30mg/kg i.p.) and perfused with 100ml of saline followed by 500 ml of ice cold 4% paraformaldehyde (pH 7.4) through the aorta. After perfusion, the part of the thoracolumbar spinal cord which contains all the TIB motoneurons (T11-L2) was removed and post fixed in 30% sucrose in 4% paraformaldehyde solution overnight. Tissues were then frozen in liquid nitrogen and stored at -80°C prior to cryostat sectioning. The tibialis anterior (TA) muscle that is largely cross-innervated by the TIB motoneurons was removed from both experimental and control limbs of each rat and weighed.

The nerve pieces that were taken out before application of fluorescent dyes were fixed in gluteraldehyde (3% in 0.1M phosphate buffer), stained with OsO_4 (3% solution in 0.1M phosphate buffer), dehydrated in ascending alcohols and embedded in araldite. Sections of 1.5um thickness were stained with methylene blue for photomicrographs and ultra-thin sections for electron micrographs were stained with uranyl acetate and lead citrate. Images of between 300 and 500 nerve fibres from randomly selected areas of each nerve cross section were photographed and saved to the JAVA image analysis system (Jandel Scientific, USA) for measurement of nerve fibre areas and perimeters. Fibre diameters, axon diameters and myelin thicknesses of the selected nerve fibres were calculated from the areas and perimeters (see Gillespie and Stein, 1983). The total number of regenerated fibres were enumerated by saving low magnification (40X) images of the whole nerve section to the JAVA system. All regenerated fibres (myelinated and unmyelinated) in each image were counted using the JAVA system.

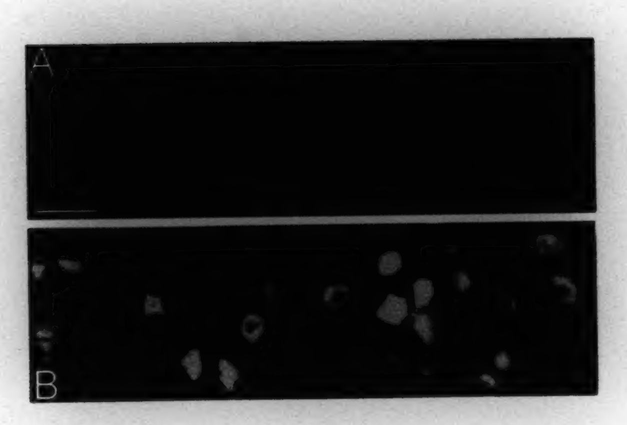
2.1.4 Motoneuron Counting

Each thoracolumbar spinal cord was mounted horizontally on a cryostat chuck with the dorsal part of the spinal cord facing up for sectioning of 50μm longitudinal sections using a freezing microtome (Jung CM 3000). Sections were serially mounted on glass slides, allowed to dry and coverslipped. Retrogradely labelled motoneurons in each spinal cord section were observed and all the motoneurons counted at 20-40X magnification under UV fluorescence at barrier filters of 430nM for FG and 580nM for FR. Cells labeled with RR could be easily identified by their red fluorescence and those labeled with FG had blue fluorescence (Figure 2). Backlabelled TIB motoneurons which regenerated their axons in the experimental hindlimb and intact TIB motoneurons were identified. Only the motoneurons in which there was a distinct nucleus and that could be visualised only under the UV fluorescent filter specific for the dye with which they were labelled were counted; multiple counting of split neurons was corrected for by the method of Abercrombie (1946).

2.1.5 Statistical Analysis

A one-way analysis of variance (ANOVA) was used to compare the mean number of TIB motoneurons that regenerated axons into the denervated distal nerve stumps after varied length of chronic denervation. Statistical significance was accepted at the 5% level ($p<0.05$). Differences between distributions were analysed using the Kolmogorov-Smirnov test to compare cumulative distribution and frequency distributions were compared using the Mann-Whitney *U* test of non-parametric distribution (Fisz,1963; see Gordon et al., 1991). The Student's *t* test was used to compare differences between mean values of fiber diameters, axon diameters and myelin thickness with statistical significance acceptable only at the 1% level ($p< 0.01$).

Figure 2.2: Photomicrographs of motoneurons backlabelled with FG (A) and FR (B) as viewed under the fluorescent microscope. Both FG and FR labeled the soma and dendrites of intact TIB motoneurons and axotomised TIB motoneurons which regenerated into the chronically denervated CP stumps. FG-labeled motoneurons were identified by their blue fluorescence and those labeled with FR by their red fluorescence. The scale bar is 30um.

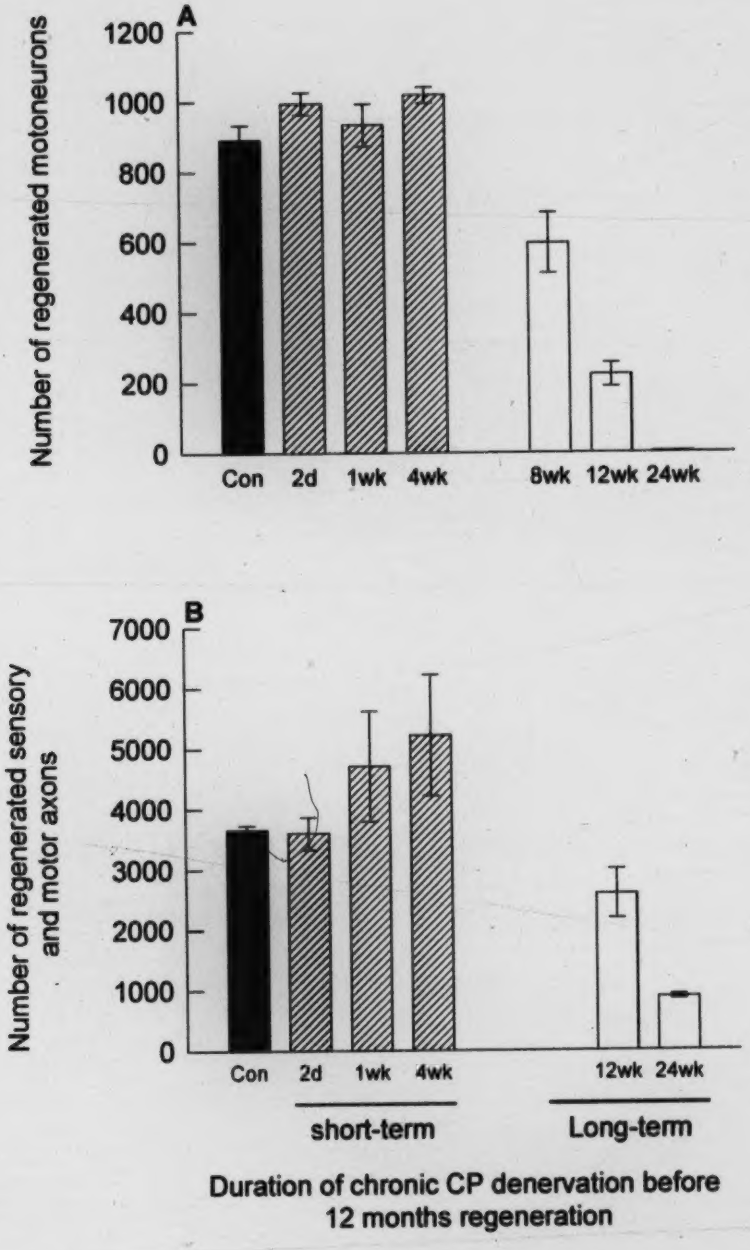

A
B

2.3 RESULTS

2.3.1 Axonal Regeneration of TIB Motoneurons into Chronically Denervated Distal CP Nerve Stump

We used retrograde fluorescent labeling to identify and count the number of freshly axotomised TIB motoneurons which regenerated their axons into chronically denervated distal CP nerve stumps, 12 months after cross-suture of the proximal TIB and distal CP nerve stumps. Chronic CP distal nerve stump denervation for up to 4 weeks month did not retard TIB motor axonal regeneration with mean numbers (± SE) varying between 994 ± 31 and 1017 ± 23 which was not statistically different from the 891 ± 42 intact TIB motoneurons counted on the contralateral side ($p>0.05$; stripped as compared to solid histograms in Figure 3A). In contrast, periods of CP nerve stump denervation which exceeded 4 weeks, severely and progressively reduced the number of freshly axotomised TIB motoneurons which successfully regenerated (clear histograms in Figure 3A). This decline in the number of the regenerated TIB motoneurons was associated with a delayed decline in the number of regenerated sensory and motor axons (Figure 3B). Interestingly, the declining ability of motoneurons to regenerate axons into the chronically denervated distal nerve stumps was associated with a delayed decline in the number of regenerated axons in the stumps, possibly associated with 1) the outgrowth of multiple axons for each TIB neuron which successfully regenerated axons into the stumps and/or 2) the contribution of regenerating axons from both motor and sensory neurons.

Figure 2.3: The numbers of TIB motoneurons that were backlabelled with either FG or RR in the spinal cord (A) and the number of regenerated axons in the distal nerve stump (B) were counted and plotted (mean ± SE) as a function of the duration of CP chronic denervation. There was no significant difference between the numbers (mean ± SE) of TIB motoneurons and axons that regenerated after short-term (2 days, 1 and 4 weeks; stripped bars) chronic denervation as compared to the number of intact contralateral TIB motoneurons and axons (filled bars) ($p<0.05$). However, there was a significant difference between the short-term denervated animal groups and the 8 week denervation time point (A) as well as between 8 weeks, 12 weeks and 24 weeks time points (A and B) ($p<0.05$). Statistical analysis was done by one-way analysis of variance (ANOVA) and the Student's *t* test.


A
Number of regenerated motoneurons
1200
1000
800
600
400
200
0
Con
2d
1wk
4wk
8wk
12wk
24wk
B
Number of regenerated sensory and motor axons
7000
6000
5000
4000
3000
2000
1000
0
Con
2d
1wk
4wk
12wk
24wk
short-term
Long-term
Duration of chronic CP denervation before 12 months regeneration

2.3.2 Fiber Diameter and Myelin Thickness

In order to determine the capacity of chronically denervated Schwann cells to remyelinate axons, we examined regenerated nerve fiber profiles at light and electron microscopic levels, 12 months after nerve cross-suture. Comparison of regenerated TIB nerve fibers after 4 and 12 weeks chronic denervation in Figure 4 indicates that regenerating axons are remyelinated by the chronically denervated Schwann cells and that the thickness of the myelin sheath depends on the duration of chronic denervation prior to reinnervation. For short-term denervation of the Schwann cells (0-4weeks), there were surprisingly high numbers of regenerated axons with smaller myelin sheaths than in normal contralateral control nerves (Figure 4B,B1). In contrast, longer durations of chronic denervation prior to regeneration were associated with larger regenerated TIB axons and thicker myelin sheaths than in normal contralateral control nerves (cf Figure 4A, A1 and C,C1). Measurements of fiber diameters showed that, indeed, the diameters of the majority of regenerated TIB nerve fibers after long-term denervation had increased whereas after short-term denervation, higher numbers of smaller diameter nerve fibers were observed (Figure 5). The size of the regenerated nerve fibers in the short-term denervated distal nerve stump varied within the same range as the contralateral control TIB nerve fibers although the distribution was skewed to the right (Figure5A-D), associated with the higher numbers of regenerating axons in the distal nerve stump per neuron (see Figure 3B). In the long-term chronically denervated stumps on the other hand, the smaller number of regenerated nerve fibers were significantly larger in size, the median shifting from 5.5 and 5.3 at 1 and 4 weeks short-term chronic denervation to 13.7 and 12.5 after long-term chronic denervation (Figure 5E, F). Increased fiber diameter in the long-term denervated nerve stumps was associated with corresponding increase in both axon diameter and myelin thickness (Figure 6) such that the normal linear relationship between myelin thickness and fiber diameter was retained because both the fiber diameters and the myelin thickness of the regenerated fibers increased (Figure 7).

2.3.3 Denervated Muscle Accept Reinnervation

The number of TIB motoneurons which regenerated axons into chronically denervated CP nerve stumps and the weight of the reinnervated TA muscles determined in the present study were compared directly in Figures 8 and 9 with the previously determined number of motor units in the reinnervated TA muscles and their isometric tetanic forces in response to stimulation of the TIB nerve (Fu and Gordon, 1995a). The correspondence between the numbers of motoneurons and reinnervated motor units as a function of duration of chronic CP nerve denervation (Figure 8) shows that it is the deterioration in the ability of the chronically denervated nerve stump to support axonal regeneration and not the inability of chronically denervated muscle to accept reinnervation, that accounts for progressive failure of functional recovery.

This conclusion is further supported by the correspondence between reinnervated muscle wet weights and tetanic forces generated by the reinnervated muscles (Figure 9). Note that both the weight and force of the reinnervated muscles decline to ~30% after 6 month chronic CP nerve denervation prior to TIB axonal regeneration (Figure 9). This decline contrasts with the much larger decline in the number of TIB motoneurons which regenerate and the number of reinnervated TA motor units (Figure 8), indicating that the rapidly progressing failure of freshly axotomised motoneurons to regenerate into chronically denervated stumps accounts for poor muscle reinnervation. In fact, motor axons which do regenerate after long-term chronic denervation, enlarge their motor units by a factor of 3-5 (Fu and Gordon, 1995b).

Figure 2.4: Light (A-C) and electron micrographs (A1-C1) of contralateral control (A,A1) TIB nerve and of regenerated TIB nerve fibers 12 months after a 4 week short-term (B, B1) and a 12 week (C, C1) period of chronic denervation of the distal CP nerve stump. Schwann cells were able to form elaborate myelin sheath around regenerated axons independent of the duration of chronic denervation. Note that the myelin thickness is decreased in B and B1, and increased in C, C1 compared to the contralateral control nerve, A, A1. Also, there are more small nerve fibers in B in contrast to the predominately large nerve fibers in C. The scale bars are 12um and 25um in A1-C1 and A-C, respectively.

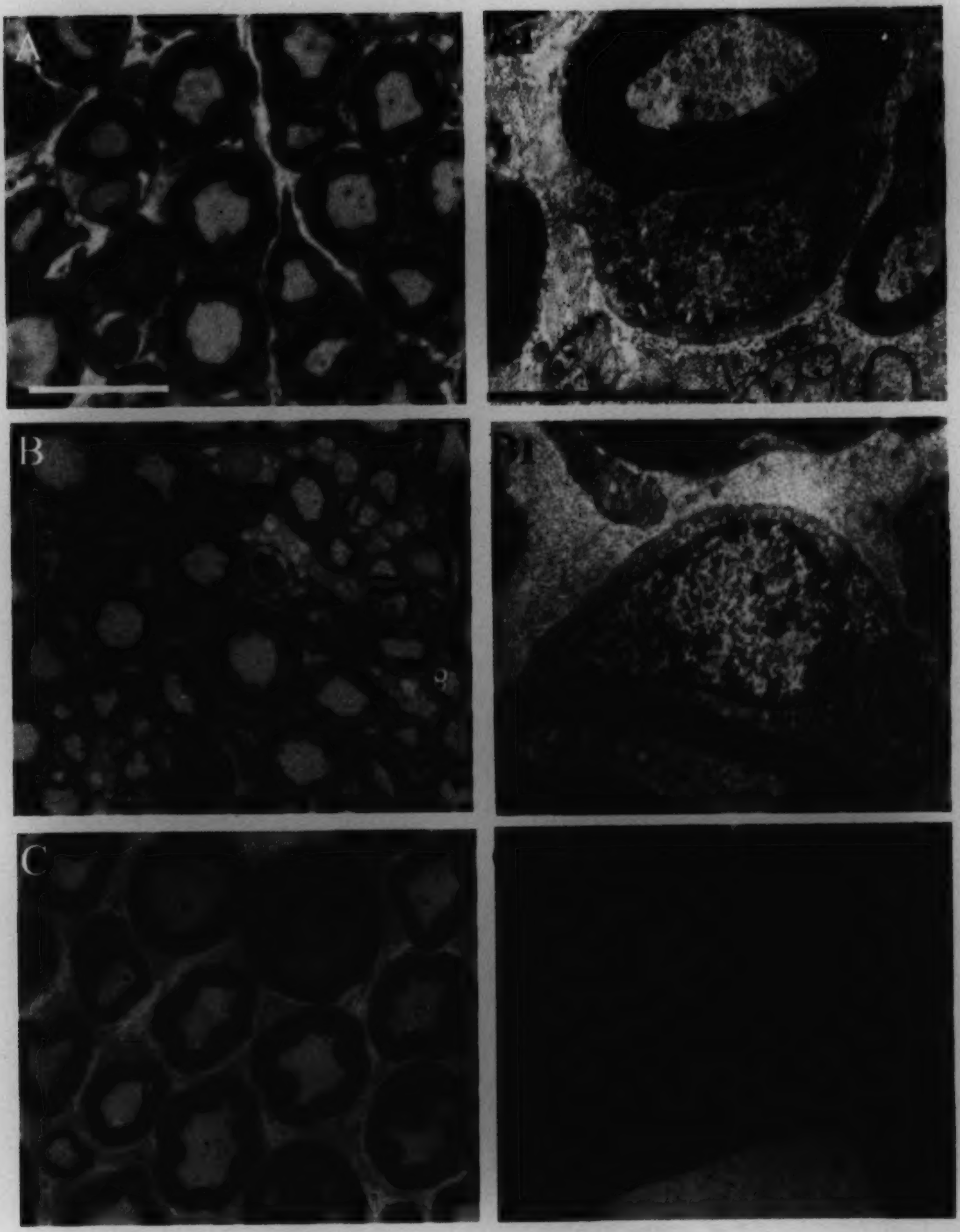

A
B
C

Figure 2.5: Comparison of the frequency histograms of contralateral control nerve fiber (A) and 12 month regenerated TIB nerve fiber diameters in the CP nerve stump after short- (B-2 days; C-1 week and D- 4 weeks) and long-term (E-12 weeks and 24 weeks) chronic denervation. There was a typical bimodal frequency distribution of fiber diameters in the control TIB nerve (A) (median is 8.23 and the two peaks are at 5.62 and 10.09) whereas this bimodal distribution was lost after chronic denervation of 1-24 weeks and 12 months regeneration. Leftward shifts of the frequency histograms towards smaller diameter fibers were observed for the short-term denervation time points (B,C and D) with median values of 6.24, 5.50 and 5.31 respectively. Mann-Whitney *U* test of these time points showed significant differences between A, B and C but no significant differences were shown between C and D ($p< 0.01$). On the other hand, rightward shifts (i.e. larger diameter fibers) of the histograms were observed at the long-term denervation time points (E, median = 13.7 and F, median = 12.5) with statistically significant differences between D and E, and between E and F (Mann-Whitney U test, $p<0.01$).

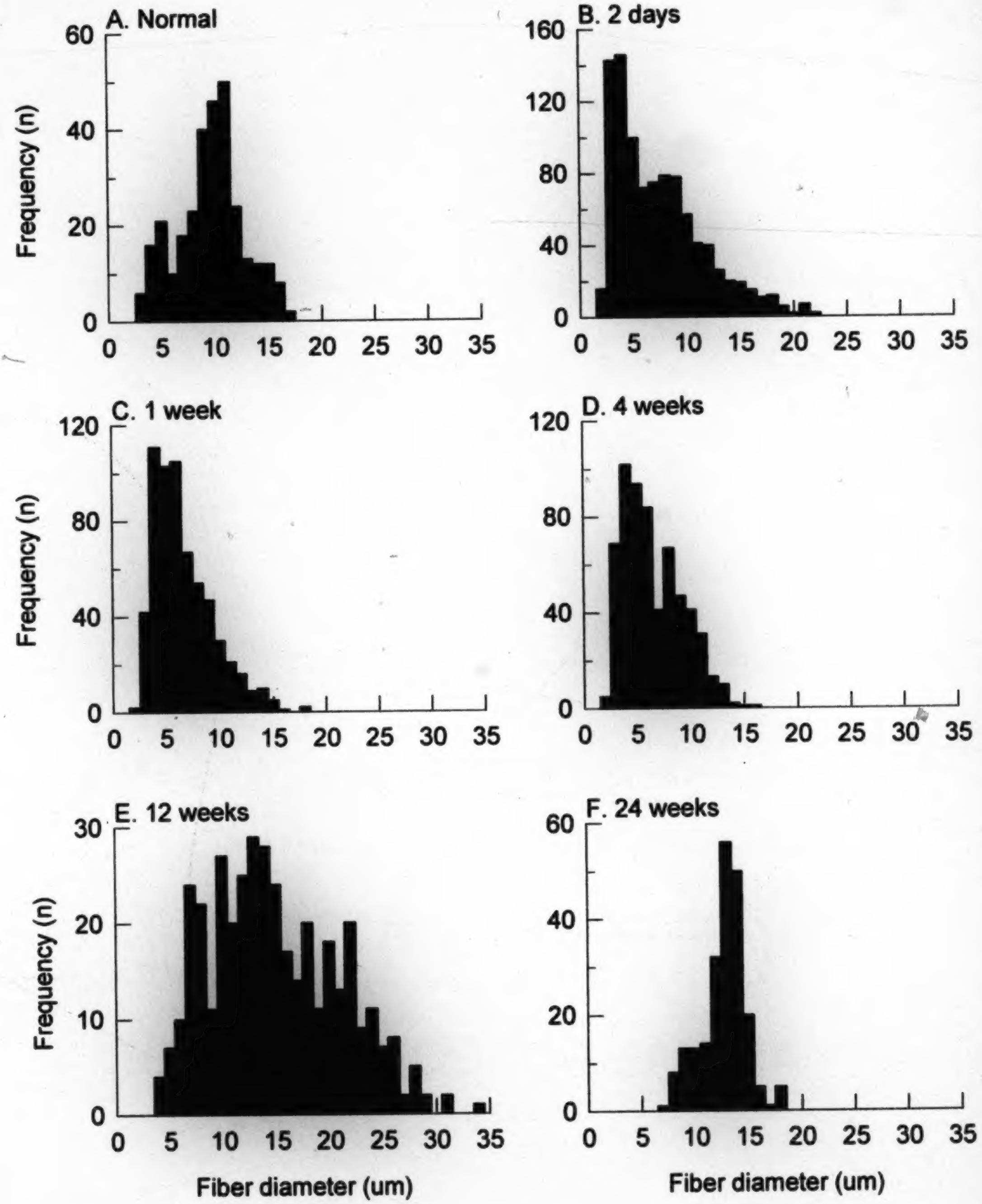

A. Normal
B. 2 days
C. 1 week
D. 4 weeks
E. 12 weeks
F. 24 weeks
Frequency (n)
Fiber diameter (um)
0
20
40
60
80
120
160
10
30
5
15
25
35

Figure 2.6: Fiber parameters of 12 month intact TIB nerves and regenerated TIB nerves plotted as a function of the duration of chronic CP nerve denervation prior to TIB-CP cross-anastomosis. The The mean ± SE of nerve fiber diameter (●), axon diameter (■) and myelin thickness (▲) are plotted. Differences between means were compared by the Student's *t* test and one-way ANOVA. Similar patterns of statistical difference were observed with the two analyses. At all time points, the means (± SEs) of the fiber parameters were significantly different from the contralateral control ($p < 0.01$) For different time points, the values for each parameter were statistically different, except between 1 week and 4 weeks ($p<0.05$).

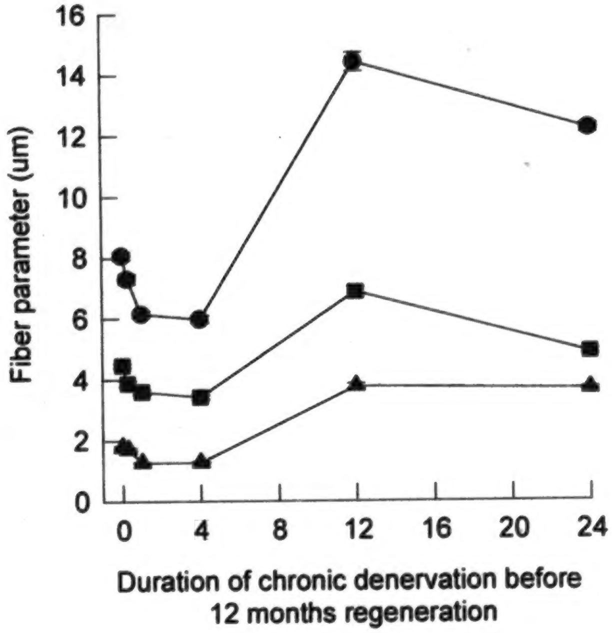

16
14
12
10
8
6
4
2
0
Fiber parameter (um)
0
4
8
12
16
20
24
Duration of chronic denervation before
12 months regeneration

Figure 2.7: The relationship between myelin thickness and fiber diameter in intact TIB nerve (A) and 12 month regenerated TIB nerves after short-(B-2 days; C-1 week and D- 4 weeks) and long-term (E-12 weeks and F- 6 24 weeks) chronic CP nerve stump denervation. The slopes of the regression lines on the double logarithmic plots did not change for either short- or long-term denervation. The slope of the regression line for the intact TIB nerve is 1.12 (regression coefficient (RO) = 0.93) and the slopes of the regression lines for the denervation time points are B, 0.93 (RO = 0.93); C, 0.87 (RO = 0.87); D, 0.95 (RO = 0.90); E, 0.94 (RO = 0.92) and F, 0.91 (RO = 0.82).

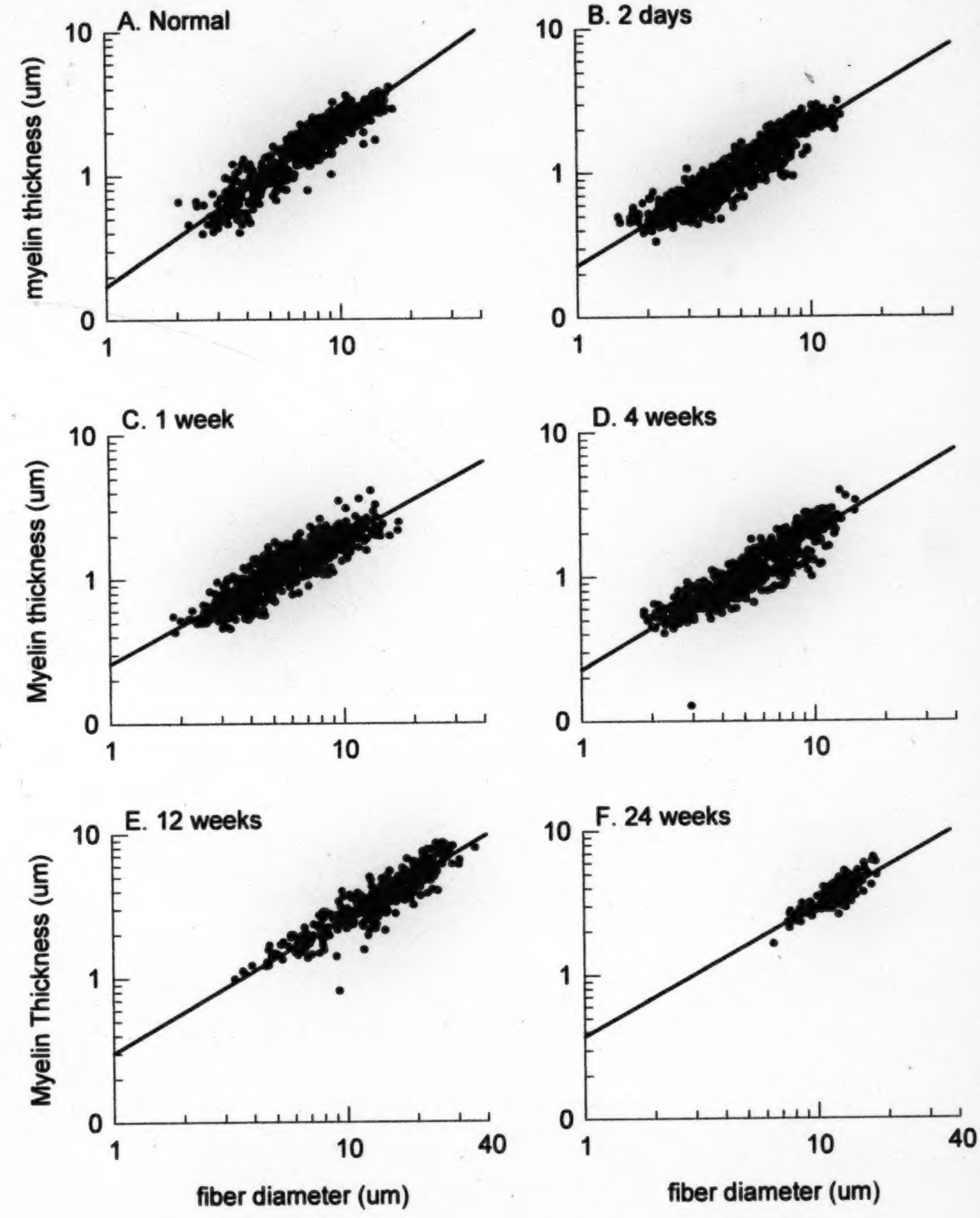

A. Normal
B. 2 days
C. 1 week
D. 4 weeks
E. 12 weeks
F. 24 weeks
myelin thickness (um)
Myelin thickness (um)
Myelin Thickness (um)
fiber diameter (um)
fiber diameter (um)
10
1
0
1
10
40

Figure 2.8: Relationships between the number of TIB motoneurons that regenerated their axons through chronically denervated CP nerve stumps (●)and the number that reinnervated denervated tibialis anterior muscle (●). There was a corresponding decline in the number of motor units (mean ± SE) as the number of regenerated motoneurons falls after long-term denervation (A). Double logarithmic plot of both values showed a direct relationship (slope = 0.30± 0.06 and regression coefficient = 0.95) (B)

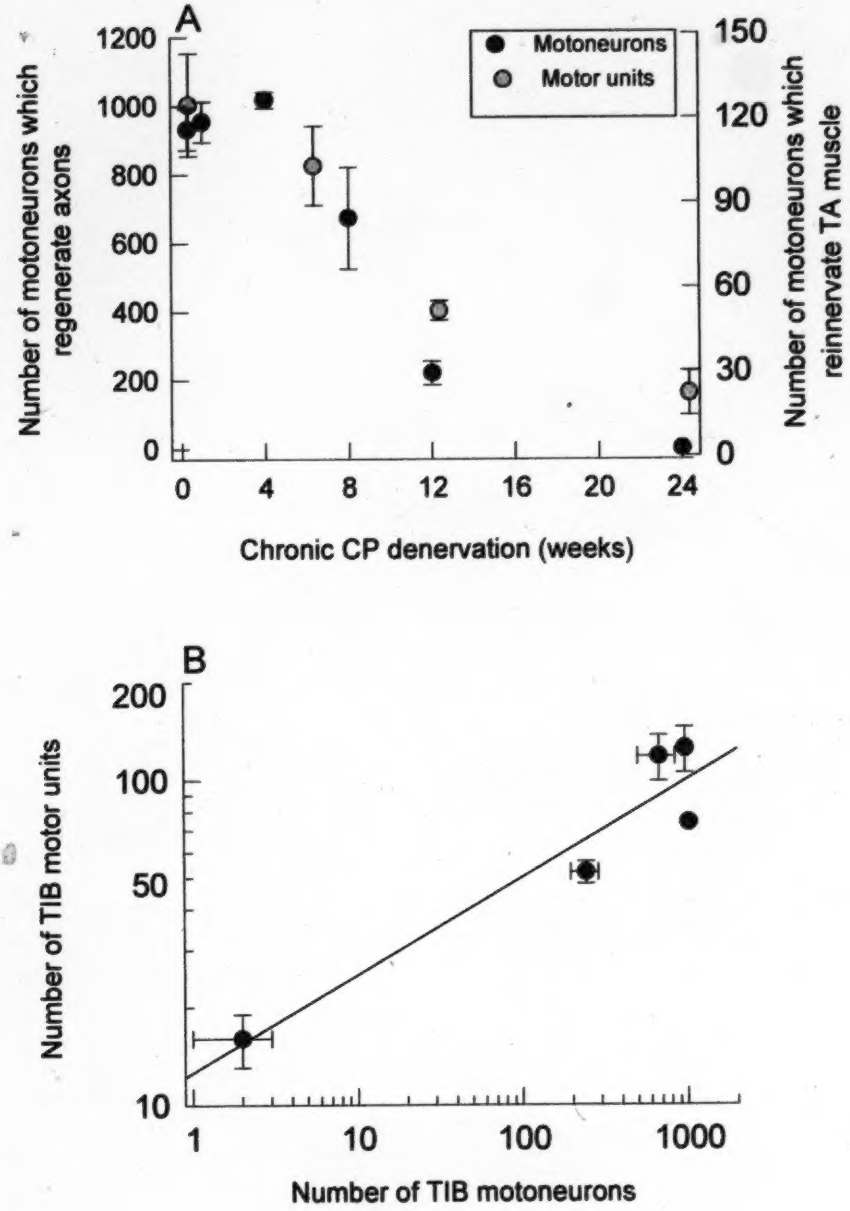
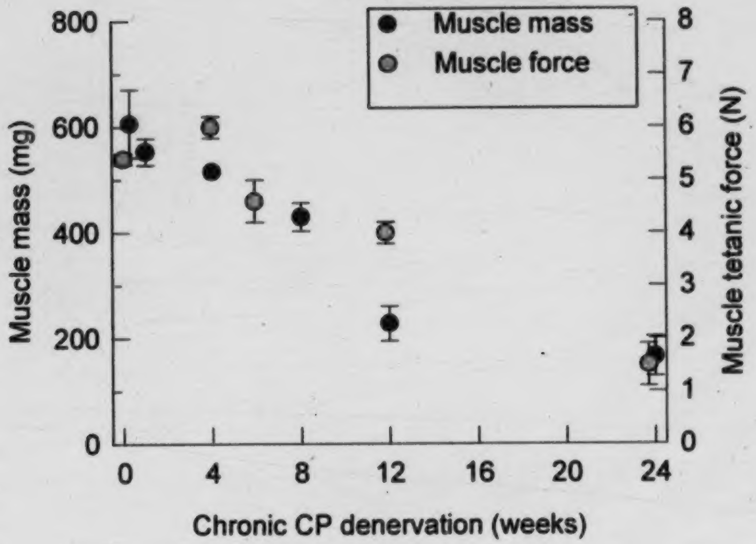
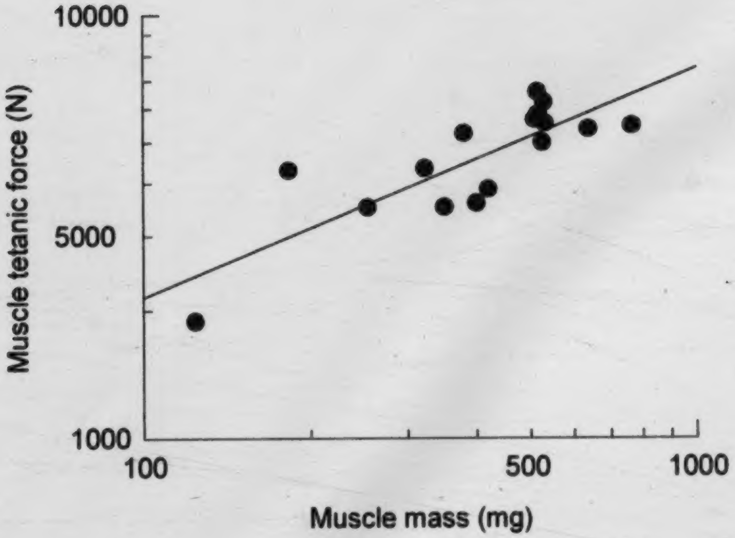

A
Motoneurons
Motor units
Number of motoneurons which regenerate axons
1200
1000
800
600
400
200
0
Number of motoneurons which reinnervate TA muscle
150
120
90
60
30
0
0
4
8
12
16
20
24
Chronic CP denervation (weeks)
B
Number of TIB motor units
200
100
50
10
1
10
100
1000
Number of TIB motoneurons

Figure 2.9: Relationship between the reinnervated TA muscle mass and its tetanic force. A reduction in the TA muscle mass was associated with a reduction in TA tetanic force (A). This was confirmed by the double logarithmic plot of the two parameters which shows a direct relationship (slope = 0.83 ± 0.21 and regression coeffeicient = 0,70) (B).


Muscle mass
Muscle force
800
600
400
200
0
Muscle mass (mg)
8
7
6
5
4
3
2
1
0
Muscle tetanic force (N)
0
4
8
12
16
20
24
Chronic CP denervation (weeks)


10000
5000
1000
Muscle tetanic force (N)
100
500
1000
Muscle mass (mg)

2.4 DISCUSSION

The results of this study demonstrate that chronic denervation of distal nerve stumps severely compromises how many axons regenerate into the distal nerve stumps and, that it is the progressive failure of the distal nerve stumps and not failure of chronically denervated muscles to accept innervation that accounts for the progressive failure of axonal regeneration with time and distance. Nonetheless, Schwann cells that survived long-term chronic denervation retain their capacity to myelinate axons and to promote recovery of their sizes.

2.4.1 Poor Axonal Regeneration into Chronically Denervated Nerve Stumps

Findings that nerve grafts lacking Schwann cells fail to support axonal regeneration demonstrates that Schwann cells are prerequisite for axonal regeneration (Hall, 1986a,b; 1997). However, how chronic denervation of Schwann cells in distal nerve stumps affects axonal regeneration and the time course of such effects, had not been described. Here, we demonstrate that long-term chronic denervation dramatically reduces regeneration of axons into the distal nerve stump (Figure 3B). Freshly axotomized and regenerating motoneurons which were sutured to chronically denervated nerve stumps progressively failed to grow in the chronically denervated nerve stumps. This failure occurs concurrent with previously reported reduction in the number of Schwann cells in the distal nerve stumps. Furthermore, this reduction provides strong evidence that the progressive reduction in number of reinnervated motor units after chronic denervation (Fu and Gordon, 1995b) is accounted for by progressive failure of axons to regenerate in the atrophic nerve stump and not an inability of chronically denervated muscles to accept axonal reinnervation (Figure 8). In fact, the correspondence between the reinnervated muscle weights and isometric forces developed by the reinnervated muscles together with the smaller decline in these parameters (to ~33% relative to immediate nerve repair; Figure 9), are consistent with the enlargement of reinnervated motor units to compensate for the poor axonal regeneration (Fu and Gordon, 1995b). The parallel decline in muscle weight and isometric force of the reinnervated muscles does not support previous views that there is an irreversible loss of the denervated atrophic muscle fibres since those muscle fibres which were reinnervated recovered muscle weight and generated force. The ability of regenerating axons to form maximally enlarged motor units indicates that long-term denervated muscle fibres survive and accept reinnervation. Recovery of reinnervated muscle fibre diameter likely involves the division and incorporation of satellite cells as they recover (Mussinin et al., 1987; Schmalbruch et al., 1991).

Although acutely denervated Schwann cells are essential for successful axonal regeneration, their capacity to support axonal regeneration was compromised by long-term chronic denervation (Figure 3). This effect on motor axonal regeneration may be extended to the sensory neurons as well, since there was a reduction in the total numbers of regenerated axons which included both sensory and motor axons (Figure 3B), although we did not estimate the number of sensory neurons which regenerated axons directly in our experiments.

The provision of trophic support for axonal regeneration by Schwann cells is associated with the changes that ensue in these cells immediately after nerve injury (Scherer and Salzer, 1996; Jessen and Mirsky, 1999; Maier et al., 1999). Following nerve section, the loss of axonal contact is associated with Schwann cell proliferation and a switch from myelinating to the non-myelinating phenotype. Myelin-associated proteins such as P0 and myelin-associated glycoprotein (MAG) are downregulated in association with the upregulation of growth-associated molecules . These include the neurotrophin receptor p75BTR, neuregulin and their receptors erb B2, erbBB3 and erbB4, glial fibrillar acidic protein, neural-cell adhesion molecule and growth-associated protein (GAP-43; Bunge, 1987; Carraway and Burden, 1995; Carroll et al., 1997; Chao et al., 1986; Chen et al., 1994; Jessen et al., 1990, 1991; Raabe et al., 1998; Rahmatullah et al., 1998; Scherer and Salzer, 1996; You et al., 1996).An important component of the non-myelinating phenotype is the expression of growth factors which include nerve growth factor, brain-derived neurotrophic factor, neurotrophin-4/5 and glial-derived neurotrophic factor (GDNF), all of which have been associated with neuronal survival and axonal growth both in the peripheral (Heumann et al., 1987a,b; Taniuchi et al., 1986; Acheson et al., 1991; Meyer et al., 1992; Fu and Gordon, 1997; Novikov et al., 1997) and central nervous systems (David and Aguayo,1981; Kierstead et al, 1989).

Regeneration of motor and sensory axons *in vivo* normally induce a second phase of Schwann cell proliferation and remyelination of the regenerated axons (Pellegrino and Spencer,1985) and sensory neurites induce Schwann cell proliferation *in vitro* (Salzer and Bunge, 1980). These mitogenic effects on Schwann cells may be mediated via the interactions between neuronally-derived neuregulin and erbB2, erbB3 and to a much less extent, erbB4 receptors expressed by Schwann cells (Carraway and Burden, 1995; Carroll et al., 1997; Li et al., 1997; Rahmatullah et al., 1998; Vartanian et al., 1997). Neuregulin induces the phosphorylation of erbB2 and erbB3 receptors and a subsequent formation of erbB2:erbB3 heterodimers which, in turn, mediates Schwann cell proliferation (Vartanian et al., 1997; Rahmatullah et al., 1998). When axonal regeneration is delayed, as is the case with our experimental model of long-term denervation of the distal nerve stump, Schwann cell proliferation is not maintained, their number decrease progressively, and their basement membrane and, hence the endoneurial tubes fragment and disappear (Giannini and Dyck, 1990; 1998; Terenghi et al., 1998; Wood et al., 1998). The progressive reduction in numbers of these long-term denervated Schwann cells correlates with the progressive decline in expression of erb B2, erb B4 after 4 weeks (Li et al., 1997) and a later decline (after 8 weeks) of p75NTR receptors in denervated Schwann cells (You et al., 1996). In contrast to erbB receptors which mediate Schwann cell proliferation, p75NTR has been implicated in mediating their apoptosis (Ferri and Bisby, 1999; Soilu-Hanninen et al., 1999). Therefore, the earlier loss of expression erbB2 and erbB4 as compared to p75NTR suggests that there is a 4 week period when apoptotic signals via p75NTR predominate in Schwann cells leading to their death and subsequent reduction in their number. Interestingly, the period between 4 and 8 weeks coincides with the time during which the number of motoneurons which regenerated axons began to decline (Figure 3A). Hence, expression of erbB2, erbB4 and p75NTR accurately reflects the capacity of Schwann cells to support axonal regeneration within 4 weeks of injury and their declining capacity with long-term chronic denervation. Therefore, the temporal decline in the expression of erbB and p75NTR receptors, fragmentation and collagenization of the endoneurial tubes as well as down-regulation of neurotrophic factors (Heumann et al., 1987; Meyer et al., 1992; Funakoshi et al., 1993; Anand et al., 1997) could explain, at least in part, the reduced axonal regeneration after long-term denervation

(>4weeks) (Figure 3).

2.4.2 Capacity of Chronically Denervated Schwann Cells to Remyelinate Regenerated Axons

Our results demonstrate that atrophic Schwann cells in chronically denervated nerve stumps supported the regeneration of progressively fewer number of axons, yet they retain their ability to respond to axonal contact by initiating myelination (Figure 3B; 4). There was also an increase in the diameter of the regenerated axons (Figure 4-6) which may be as a result of the effect of the size of muscle target and Schwann cells both of which influence axonal diameter (Gordon and Stein, 1982; Aguayo and Bray, 1984). The reduced number of motoneurons which regenerate axons into 12 week chronically denervated distal nerve stumps formed enlarged motor units (Fu and Gordon, 1995b) which exposes them and their axons to the influence of more target tissue. Previous experiments showing that increasing the size of innervated target increases the size and myelination of the axons (Voyvodic, 1989) and that exogenous GDNF increased nerve fibre conduction velocity in axotomised motoneurons suggest that GDNF and possibly other neurotrophins, may act as the target-or Schwann cell-derived factors which regulate axonal diameter (Munson and McMahon, 1997).

Insights into the molecules involved in the regulation of axonal diameter has shed more light on the ways in which muscle target and Schwann cells myelination may regulate axonal diameter. The size of axons is regulated by both the level of neurofilament expression and the phosphorylation of the lysine-serine-proline (KSP) repeats of the medium (NF-M) and heavy (NF-H) molecular weight isoforms of the protein (Hirokawa et al., 1984; Hoffman et al., 1985; Hoffman et al., 1987; Bisby and Tetzlaff, 1992; reviewed by Bisby and Tetzlaff,1992; Nakagawa et al.,1995). Neurofilament expression is downregulated after axotomy and recovers only if regeneration is permitted (Goldstein et al., 1988; Hoffman and Cleveland, 1988; Tetzlaff et al.,1988a, b; Petrov et al., 1996). However, some recovery of neurofilament synthesis begins before axons reach their targets (Tetzlaff et al., 1988a, b), suggesting that endoneurial factors within the distal nerve stump regulate neurofilament expression in the neurons. Phosphorylation of NF-M and NF-H is locally regulated by myelinating Schwann cells such that axonal diameters are larger at internodal as compared with nodal areas (Carden et al., 1987; Hsieh et al., 1994). The failure to form compact myelin in Trembler mice due to the absence of peripheral myelin protein PMP22, is associated with reduced NF phosphorylation and axonal caliber, in spite of the normal level of expression of neurofilament (Low, 1976a, b; de Waegh et al., 1992; Suter et al.,1992a, b). In Shiverer mice, where central myelin is also not compacted due to knock-out of myelin basic protein, small nerve fibres have also been associated with lack of compact myelin and reduced NF phosphorylation (Roach et al., 1985; Brady et al., 1999). The most direct evidence linking myelination to neurofilament phosphorylation and, in turn, to axonal size is that MAG knock-out mice have reduced phosphorylation of neurofilament protein and axonal size (Yin et al.,1998).

Both neurofilament expression and phosphorylation are linked to myelination in central axons (Brady et al.,1999). However, neurofilament expression in peripheral axons, unlike phosphorylation, does not depend on myelination but strongly depends on target reconnection (de Waegh et al., 1992). For example, target connection completely reverses the decline in axonal diameter of axotomised motoneurons (Gordon and Stein, 1982) consistent with normalisation of level of neurofilament expression after target reinnervation (Hoffman and Cleveland, 1988; Tetzlaff et al., 1988a, b). Therefore, it seems that the primary influence on the diameter of regenerated nerve fibres after long-term chronic denervation is the size of the muscle target they reinnervate. These regenerated nerve fibres are able to induce Schwann cell myelination and their ultimate size is probably determined by the axon-Schwann cell cross-talk, by way of the influence of Schwann cell myelination on phosphorylation of neurofilament proteins in the axons. This is reflected in the sustained linear relationship we observed between the myelin thickness and the nerve fibre diameter of regenerated nerve fibres after long-term chronic denervation (Figure 8).

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CHAPTER 3

3.0 LACK OF UPREGULATION OF GLIAL-DERIVED NEUROTROPHIC FACTOR CORRELATES WITH POOR AXONAL REGENERATION AFTER LONG-TERM SCHWANN CELL DENERVATION

Adapted from the original publication:

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3.1 INTRODUCTION

The Schwann cells in the distal stumps of injured peripheral nerves have been suggested to assume the role of "substitute target" after injury. This is because changes in their gene expression after injury include upregulation of mRNAs for growth factors and their receptors, and cytokines, some of which have been shown to be important for survival of motoneurons (Scherer and Salzer, 1996; Fu and Gordon, 1997). Glial cell line-derived neurotrophic factor (GDNF) is one of the neurotrophic factors that are upregulated in the distal stump of injured peripheral nerve and denervated muscle (Trupp et al., 1995; Naveilhan et al., 1997). It was discovered by its capacity to promote dopamine uptake and survival of central dopaminergic neurons (Lin et al., 1993). It is retrogradely transported to motoneurons (Yan et al., 1995) and has been shown to be very potent in promoting survival of cultured developing motoneurons (Henderson et al., 1994); it accelerated initial nerve regeneration after a crush injury when applied exogenously (Naveilhan et al., 1997).

The upregulation of regeneration-associated genes (RAGs) and Schwann cell proliferation in the distal stumps of injured nerves are very transient after the injury (Fu and Gordon, 1997). Likewise, the upregulation of GDNF expression after nerve injury in the distal nerve stumps is not maintained (Naveilhan et al., 1997; Hoke et al., 2000). A second phase of Schwann cell proliferation is induced when distal nerve stumps are reinnervated by regenerating axons after immediate nerve repair (Pellegrino and Spencer, 1985) similar to the effect of sensory neurites on Schwann cells *in vitro* (Salzer and Bunge, 1980). However, whether this secondary response of Schwann cells is accompanied by upregulation of RAGs is not known.

Recently, we showed that if regeneration of axons into the distal nerve stumps was delayed beyond 4 weeks, thus denying the neurons quick assess to the upregulated RAGS, their capacity to regenerate axons into the distal nerve stump is compromised (Sulaiman and Gordon, 2000). This may be due to the reduced capacity of the long-term chronically denervated Schwann cells to respond to regenerating axons by proliferation and upregulation of RAGs, which ultimately results in poor motor axonal regeneration. This project focuses on the expression of GDNF and its receptor subunits (GFRα-1, GFR-α-2, GFRα-3 and RET) in the distal nerve stumps as a duration of chronic denervation and during reinnervation of distal nerve stumps that have been subjected to short-(4 weeks) or long-term chronic denervation. We hypothesised 1) a progressive decline in the levels of expression of GDNF and its receptors with increase in the duration of chronic denervation, 2) freshly cut regenerating axons sutured into short-term denervated distal nerve stumps may induce the re-expression of GDNF and GFRα receptors by Schwann cells in these nerve stumps thereby sustaining the growth permissive environment for these axons, 3) long-term denervated Schwann cells have reduced responsiveness to the re-induction of mRNA for GDNF and GFRα receptors by the regenerating axons and this is responsible, at least in part, for the reduced capacity of long-term chronically denervated Schwann cells to support motor axonal regeneration we observed in our previous experiments (Fu and Gordon, 1995; Sulaiman and Gordon, 2000).

We found that i) upregulation of GDNF was not maintained after long-term chronic denervation (> 3 months) despite the sustained upregulation of its GFRα-1 and GFRα-2 receptors, the RET and GFRα-3 were not detected in the distal nerve stumps; ii) regeneration of axons for 11 or 21 days in the short-term chronically denervated distal nerve stumps increased GDNF mRNA levels in the nerve stumps to a level similar to the GDNF upregulation after the initial denervation. In contrast, the GDNF mRNA levels in the distal nerve stumps 11 or 21 days after axonal regeneration in the long-term chronically denervated distal nerve stumps were very low and similar to the levels seen in chronically denervated sciatic nerves.

3.2 MATERIALS AND METHODS

3.2.1 Animal Surgery

All experimental protocols involving animals were reviewed and approved by the Universities of Calgary and Alberta animal care committees following the Canadian Council of Animal Care guidelines. Adult male Sprague-Dawley rats (200-300 g) were used to create a model of chronic denervation. Briefly, a unilateral sciatic nerve transection was done at mid thigh, 1 cm above the sciatic trifurcation using aseptic surgical techniques under anesthesia (pentobarbital 65 mg/kg ip). The distal end of the proximal sciatic nerve was trimmed about 3mm in order to prevent regeneration into the cut distal segment at the time points we studied. Sham-operated contralateral sciatic nerves that were exposed but not injured were used as controls. In delayed coaptation experiments a well-characterized model of regeneration into long-term denervated nerve stumps was used (Fu and Gordon, 1995b; Li, 1997; Sulaiman and Gordon, 2000). Adult male Sprague-Dawley rats received a unilateral transection of the tibial (TIB) branch of the sciatic nerve. The proximal end of the TIB nerve was prevented from regenerating into the distal segment by deflecting it cranially and then suturing it to a nearby muscle. After 1 month (short-term) or 6 months (long-term) of denervation, a fresh transection of the common peroneal (CP) branch of the sciatic nerve was done and the proximal end of this branch was resutured into the distal end of the previously denervated TIB nerve to encourage axonal regeneration of the freshly axotomized CP neurons into the denervated TIB nerve stumps for 11 or 21 days . Five-15 animals were used in each group at each time points in the study.

3.2.2 RNA and Protein Studies

At 48 hours, 1 week, 1, 3 and 6 months a segment of the sciatic nerve distal to the transection and a segment from corresponding contralateral side were harvested for RNA and protein studies. Similarly in the regeneration studies, the segment of the tibial nerve distal to the resuture site containing the regenerating CP nerve axons and a segment from the comparable contralateral side were harvested on dry ice. Total RNA was isolated with TRIzol reagent (Life Technologies, Burlington, Ontario, Canada) (Chomczynski et al., 1987) and reverse transcribed using random hexamer primers. Semiquantitative multiplex PCR was done using the "primer-dropping" technique (Wong et al., 1994) with primers for the gene of interest and an internal control, GAPDH, in the same reaction tube. Primer sequences for GDNF, GFRα-1, GFRα-2, GFRα-3 and RET were designed from mRNA sequences deposited in Genbank and have been described previously (Hoke et al., 2000). Primer sequences for GAPDH were published previously (Wong et al., 1994). These primer pairs were optimized to remain in the linear phase of the PCR reaction at the cycle numbers used. PCR cycle parameters were as published (Hoke et al., 2000). PCR products were electrophoresed in 2% agarose gel and bands were visualized with ethidium bromide. Image analysis was done using NIH Image for Macintosh (Version 1.6.1, http://rsb.info.nih.gov/nih-image/). Density of the band for the gene of interest was adjusted for the density of the band of the GAPDH, and compared to that of the sham-operated side. Statistical analysis was done using a 3 factor split plot analysis of variance corrected for multiple comparisons (the critical alpha level set at $p=0.005$) in Statview program for Macintosh (version 5.0.1, SAS Institute Inc.). Appropriate controls including omission of the reverse transcriptase enzyme were used to exclude genomic DNA amplification.

Fresh frozen distal nerves were homogenized and solubilized in RIPA buffer with protease inhibitors (Canning et al., 1996) for the determination of GDNF protein content. GDNF protein content was measured using standard ELISA techniques with anti-GDNF antibody (Santa Cruz, CA) and normalized to the amount of total protein in the samples. Recombinant human GDNF (gift from Amgen Inc., Thousand Oaks, CA) was used to create the standard curve in the ELISA assays. Measurements were done in triplicates.

3.2.3 Immunohistochemistry

For immunohistochemical studies the animals were anesthetized and perfused with 4% paraformaldehyde under general anesthesia. The distal sciatic nerves were harvested and post-fixed with 4% paraformaldehyde at 4°C for another 4-8 hours. The tissue was cryoprotected in 15% followed by 30% sucrose, and sectioned at 7 μM using a cryostat. Standard immunohistochemistry was done using the anti-GDNF antibody.

3.3 RESULTS

3.3.1 Changes in GDNF Expression with Chronic Denervation

GDNF mRNA was expressed at very low levels in the sham-operated control sciatic nerves. However, there was a significant upregulation of GDNF mRNA in the distal segment of transected sciatic nerves, as early as 48 hours (Figure 1). This upregulation of GDNF mRNA was not sustained with chronic denervation beyond 3 months and GDNF mRNA levels were back to baseline by 6 months (Figures 1 and 2). The changes in GDNF protein level in the denervated sciatic nerve paralleled the changes in the mRNA levels. Using ELISA we showed that the GDNF content of a sham-operated sciatic nerve was 0.17 ng per 1 mg/ml of total protein. At 1 month, the GDNF protein content in the denervated sciatic nerve was increased by almost 3 folds to 0.53 ng per 1 mg/ml of total protein. However, by 6 months the levels in the denervated sciatic nerve were back to baseline at 0.18 ng per 1 mg/ml of total protein. These results are the average of 3 separate samples and the level of GDNF protein at 1 month of denervation was statistically significantly different from the sham-operated or 6 month-denervated sciatic nerves. Similarly, the upregulation in protein content could be observed with indirect immunohistochemistry (Figure 3).Taken together, these findings suggested that the Schwann cells of the sciatic nerve respond to denervation or loss of axonal contact by upregulating their expression of GDNF but the expression is not maintained.

The mRNA levels of the binding receptors for the GDNF family of growth factors, GFRα-1 and GFRα-2 showed a relative delayed upregulation after denervation (Figures 4 and 5). In contrast, there were no significant changes in the mRNA levels of the GFRα-3, which does not bind GDNF, during chronic denervation (data not shown for simplicity). RET mRNA levels were basically unchanged throughout the course of the study (Figures 4 and 5).

Effects of Delayed Regeneration on GDNF Expression

In order to test the hypothesis that long-term chronic denervation results in irreversible changes in Schwann cells that hamper regenerative efforts of neurons, we used delayed coaptation model of sciatic nerve injury. We picked an early time point (1 month) when motor axonal regeneration is still robust with delayed coaptation and a late time point (6 months) when regeneration is very poor (Fu and Gordon, 1995b; Sulaiman and Gordon, 2000) to examine changes in gene expression of GDNF. Freshly cut CP nerves were sutured to previously denervated TIB nerves and the nerves distal to the suture site were collected after 11days when we expect to see a large upregulation of GDNF expression associated with axonal ingrowth. The GDNF mRNA levels of the distal nerve 11days after early coaptation (ie 1 month of denervation) were similar to the GDNF upregulation after the initial denervation (Figure 6). The presence of regenerating axons did not induce any further increase in the GDNF mRNA expression at 11 days after copatation. In contrast, the GDNF mRNA levels 11days after late coaptation (ie 6 months of denervation) were very low and similar to the levels seen in chronically denervated sciatic nerves (Figure 6). This suggested that the Schwann cells of the distal tibial nerve which were denervated for a prolonged period of time were unable to upregulate GDNF mRNA levels when they were challenged with the regenerating axons of the freshly transected CP nerve.

Figure 3.1: Changes in the GDNF mRNA levels in denervated sciatic nerve as measured by semi-quantitative multiplex PCR at different lengths of chronic distal nerve stump denervation. (SH: sham-operated control side, TR: transected sciatic nerve). GDNF mRNA was rapidly upregulated 48 hours after nerve transection, reaching a peak at 1 week prior. There was sustained expression until 3 months after nerve transection but by 6 months, GDNF expression has been reduced to minimum.

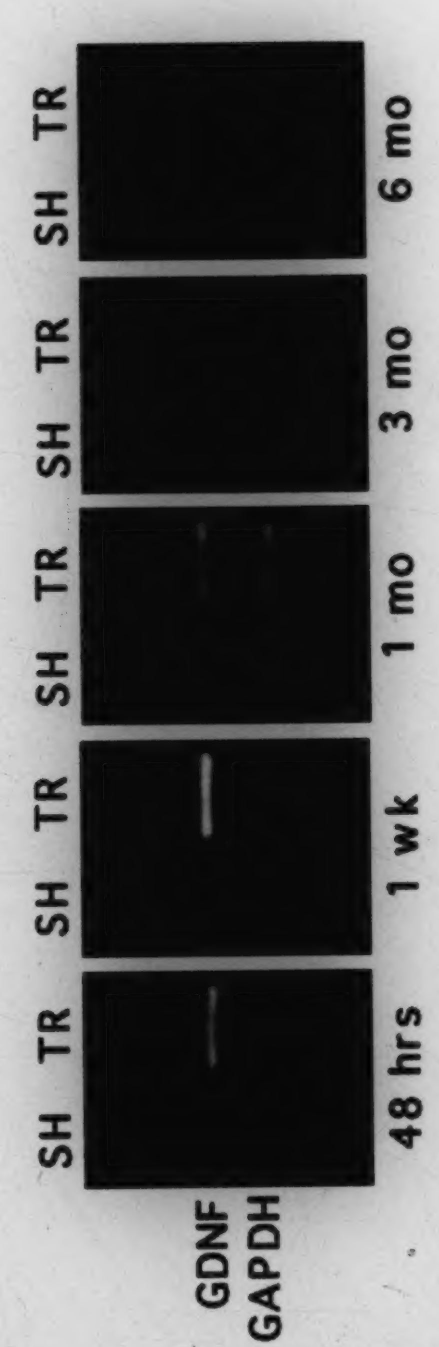

SH TR
SH TR
SH TR
SH TR
SH TR
48 hrs
1 wk
1 mo
3 mo
6 mo
GDNF
GAPDH

Figure 3.2: Changes (mean ± SE) in the levels of GDNF expressions plotted as a function of duration of chronic denervation of the distal nerve stumps. Note peak expression of GDNF at 1 week and its minimal expression at 6 months.

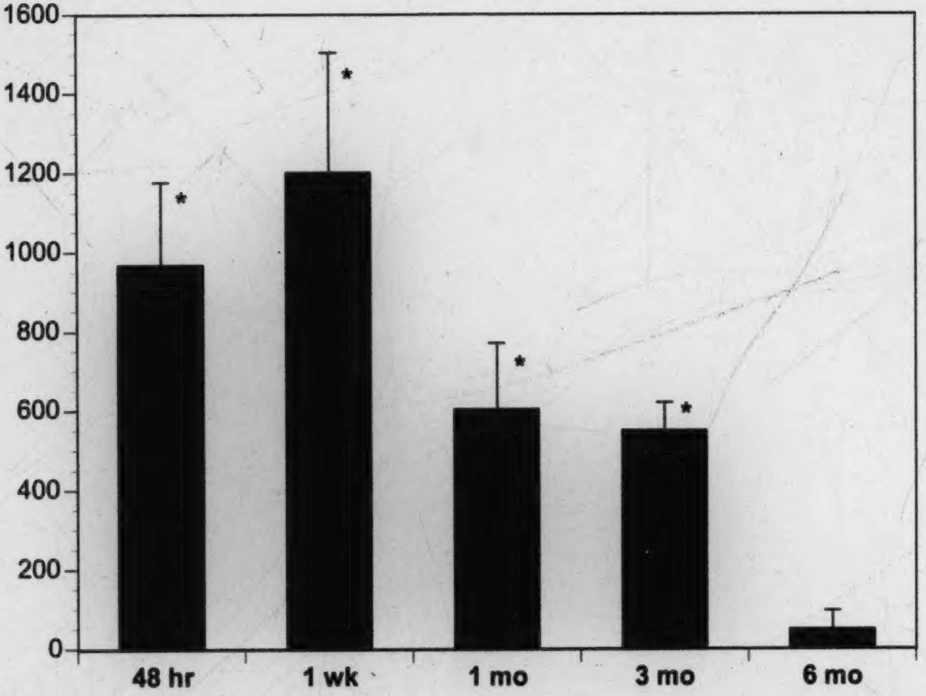


Figure 2: GDNF mRNA levels expressed as a percent change in transected over sham-operated nerves (* $p < 0.005$)

Figure 3.3: Photomicrographs showing immunohistochemical staining of GDNF protein expression in nerves one week after transection compared to sham-operated nerves. Note the intense staining of the 1-week denervated nerves depicting high level of GDNF protein in these nerves.

Figure 3.4: Changes in the GFRα-1, GFRα-2 and RET mRNA levels in 1 month denervated sciatic nerve as measured by semi-quantitative multiplex PCR. (SH: sham-operated control side, TR: transected sciatic nerve). Both GFRα-1 and GFRα-2 are upregulated with GFRα-1 being expressed at a higher level than GFRα-2 as evidenced by the higher intensity of GFRα-1 staining. RET mRNA was not detected in the distal nerve stump.

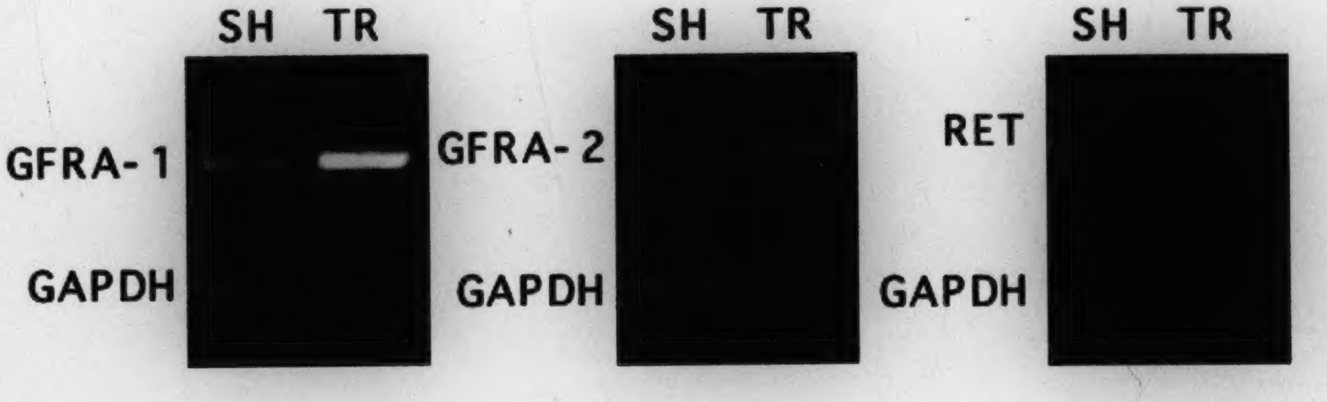

SH
TR
GFRA- 1
GAPDH
SH
TR
GFRA- 2
GAPDH
SH
TR
RET
GAPDH

Figure 3.5: Levels of expression of the GDNF receptors GFRα-1, GFRα-2 and RET plotted as a function of duration of chronic denervation. GFRα-1 was upregulated at 1 week post transection whereas GFRα-2 was not upregulated until 3 weeks later, after which the higher levels of expression of both receptors are maintained even after 6 months chronic denervation. GFRα-1 peaked at 3 months and return to the comparable levels of expression with GFRα-2 afterwards; RET mRNA level was not detectable throughout the duration of chronic denervation.

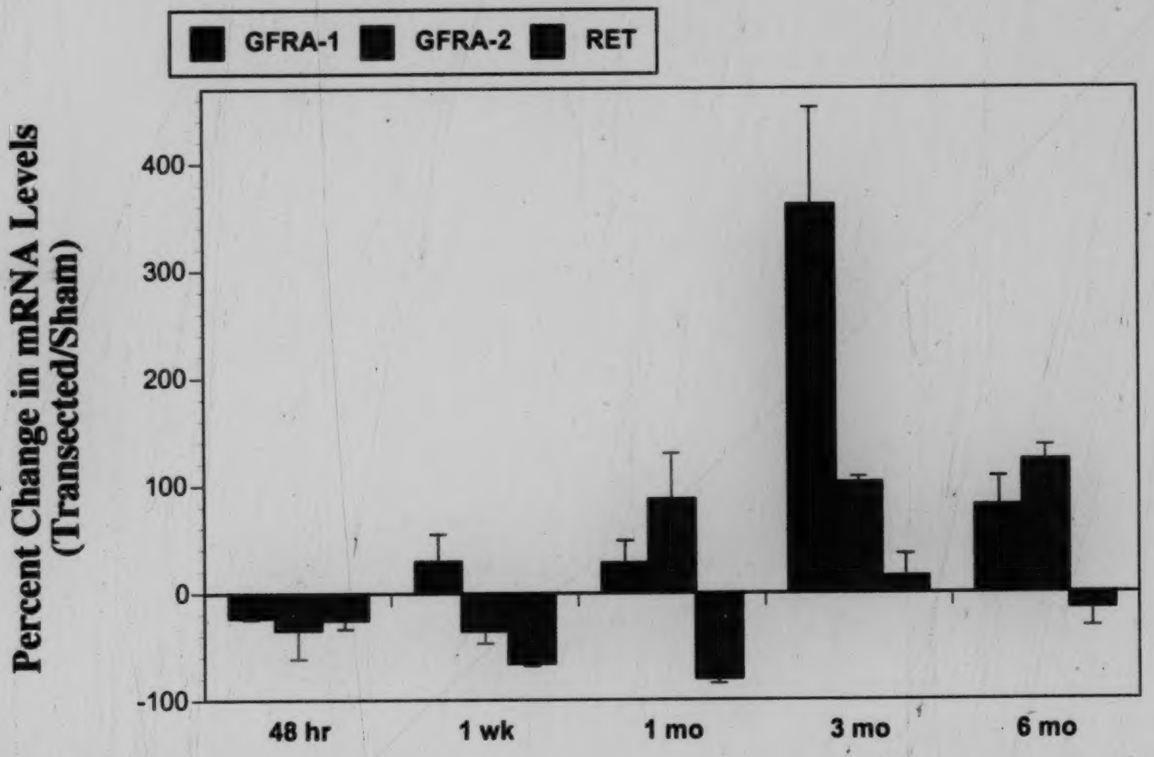
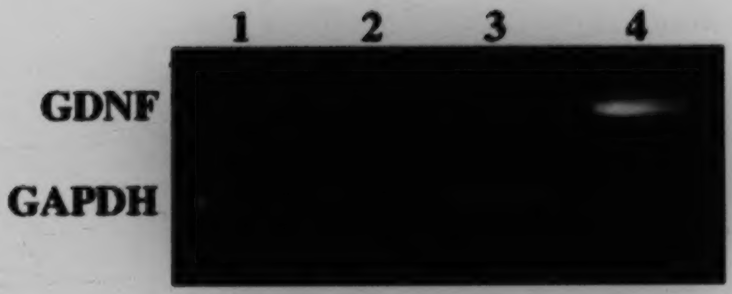


Figure 5: GFRα-1, GFRα-2 and RET mRNA levels expressed as a percent change in transected over sham-operated nerves

Figure 3.6: Changes in the GDNF mRNA levels in 6 month denervated nerve and in tibial (TIB) nerve after short- and long-term delayed coaptation. Lane 1: Sham-operated control sciatic nerve; Lane 2: 6 month denervated nerve with no coaptation; Lane 3: 11 days after delayed coaptation of freshly cut common peroneal (CP) nerve and long-term (6 months) chronically denervated distal tibial nerve stumps; Lane 4: 11 days after delayed coaptation of freshly cut CP nerve and short-term (1 month) chronically denervated distal tibial nerve stumps. CP-TIB coaptation after short-term TIB chronic denervation induced upregulation of GDNF whereas coaptation after long-term TIB chronic denervation failed to induce GDNF expression.


1
2
3
4
GDNF
GAPDH

3.4 DISCUSSION

3.4.1 Schwann Cell Response to Nerve Injury Supports Peripheral Nerve Regeneration

The Schwann cells of the nerve stump distal to the site of injury are essential for successful axonal regeneration and reinnervation of denervated target (Hall, 1986a, 1986b). Immediately after nerve injury, the Schwann cells which were previously associated with myelinated axons, enter the proliferative phase and other changes which they undergo including changes in their gene expression reflect their phenotypic changes (Scherer and Salzer, 1996; Jessen and Mirsky, 1999; Maier et al., 1999). Molecules which are important for their capacity to myelinate axons such as P0 and myelin-associated glycoprotein (MAG) are downregulated and regeneration-associated molecules like the low-affinity nerve growth factor receptor p75NTR, neuregulin and their receptors erb B2, erbB3 and erbB4, glial fibrillar acidic protein, neural-cell adhesion molecule and growth-associated protein (GAP-43) are upregulated (Bunge, 1987; Chao et al., 1986; Jessen et al., 1990; Chen et al., 1994; Carraway and Burden, 1995; Scherer and Salzer, 1996; You et al., 1997; Carroll et al., 1997; Raabe et al., 1998; Rahmatullah et al., 1998). In addition, the distal nerve stump assume the role of 'temporary targets' for neurons which regenerate their axons as growth factors which are normally target-derived, such as nerve growth factor, brain-derived neurotrophic factor, neurotrophin-4/5 and glial-derived neurotrophic factor (GDNF) are also upregulated in the distal nerve stumps (Taniuchi et al., 1986; Heumann et al., 1987; Acheson et al., 1991; Meyer et al., 1992; Fu and Gordon, 1997; Novikov et al., 1997).

However, the trophic environment created by the Schwann cells of the distal nerve stumps is not sustained indefinitely as the upregulated regeneration-associated molecules are downregulated and the mitotic activity of Schwann cells is lost with time (Fu and Gordon, 1997; Terenghi et al., 1998; Wood et al., 1998). In these experiments, we demonstrate the rapid upregulation of GDNF after nerve injury and its progressive downregulation as Schwann cells remained denervated for prolonged period of time. By 3 months, the expression of GDNF is significantly reduced and hardly detectable after 6 months. Concurrent with the upregulation of GDNF, there was delayed upregulation of 2 of its receptors, GFRα1, GFRα2 with GFRα1 being upregulated at 1 week followed by GFRα2 upregulation at 1 month. No changes were observed in the levels of expression of the RET and GFRα3 GDNF receptors, similar to what have been previously reported in mice sciatic nerve (Naveilhan etal., 1997).

3.4.2 GDNF Expression after Nerve Injury and During Axonal Regeneration

GDNF and related factors neurturin, artemin, and persephin are members of the GDNF family of neurotrophic factors, which is a new subgroup in the transforming growth factor-β superfamily (reviewed by Airaksinen et al., 1999). GDNF interacts with a multi-subunit receptor system. The glycosylphosphatidyl inositol (GPI) membrane-linked receptor subunit, called GFRα, has been described as the ligand-binding domain whereas the RET tyrosine kinase receptor was identified as the signalling component of the GDNF receptor (Durbec et al., 1996; Jing et al., 1996; Trupp et al., 1996) although RET-independent signaling of GDNF via GFRα1 has been reported (Ylikoski et al., 1998). In most cells which are responsive to GDNF, the association of GDNF with the GFRα appears to be required for

RET binding and activation. In fact, a recombinant soluble form of GFRα, lacking the C-terminal hydrophobic GPI domain, has been shown to mediate RET binding and activation by GDNF (Jing et al., 1996; Treanor et al., 1996).

Experiments on mice by Naveilhan and co-workers (1997) reported rapid upregulation of mRNA levels of GDNF and GFRα, in the distal nerve stump after sciatic nerve injury and upregulations of RET receptor in the proximal stump of the injured nerve, DRG neurons and spinal cord motoneurons. They suggested that expression of GFRα by Schwann cells in the distal nerve stump, concentrates and presents locally produced GDNF to regenerating axons, similar to the role of neurotrophin receptor, p75NTR. Production of a soluble form of GFRα by Schwann cells may also serve to present GDNF-GFRα complexes to regenerating axons, which can bind directly to the RET receptor on these axons (Naveilhan et al., 1997). We did not observe an upregulation of the RET receptor in the distal nerve stumps but GDNF and both GFRα1 and GFRα2 receptors were upregulated consistent with the observations in mice. Therefore, the similar pattern of expression of GDNF and its receptors in mice and rat suggest that the hypothesized roles of the GDNF receptors may be applicable to both species. Interesting, the upregulation of the GFRα receptors was not until after the peak upregulation of GDNF at 1 week and their levels of expression were relatively high even after GDNF expression was downregulated to a minimal level (figures 2 and 5). Taken together, these observations indicate that upregulation of the GFRα receptors require maximal induction of GDNF but once upregulated their levels of expression are maintained even after long-term chronic denervation.

3.4.3 The Detrimental Effect of Long-term Schwann Cell Denervation on Motor Axonal Regeneration May Be Mediated by Lack of GDNF Upregulation

We found that the expression of GFRα receptors is maintained in the distal nerve stumps even after long-term chronic denervation when GDNF expression is reduced to minumal level (figure 5). This suggests that a major deterrent to optimal axonal regeneration in the distal nerve stumps is the untimely downregulation of GDNF, a potent neurotrophic factor for the regenerating motoneurons either alone or as a GDNF-GFRα complex presented to the growth cone. When axons regenerated into short-term (1 month) chronically denervated distal nerve stumps, GDNF expression in the reinnervated nerve stumps was re-induced to the same level as seen immediately after nerve injury (figure 6, lane 4). However, when axons regenerated into 6 month chronically denervated (long-term) distal nerve stumps in which the expression of GFRα is high, GDNF expression was not re-induced (figure 6, lane 3). Therefore, freshly axotomized motoneurons which regenerated axons into 6 month chronically denervated nerve stumps lack the trophic support of GDNF and this, at least in part, probably account for the reduced capacity of these motoneurons to regenerate axons (Sulaiman and Gordon, 2000).

Changes in the gene expression of Schwann cells after nerve injury is associated with their proliferation (Scherer and Salzer, 1996). Likewise, the observed upregulation of GDNF expression in the distal nerve stumps upon reinnervation after short-term chronic denervation is not an isolated event as other events such as Schwann cell proliferation and their conversion to the myelinating phenotype have been reported (Pellegrino and Spencer, 1985). Neuregulins secreted by the growth cone induce phosphorylation of, and formation of heterodimers by erbB2 and erbB3 receptors on Schwann cells thereby inducing their proliferation (Vartanian et al., 1997; Rahmatullah et al., 1998). After long-term denervation, Schwann cells lose their expression of erb receptors (Li et al., 1997) which probably abolishes their proliferative response to regenerating axons, an effect of long-term chronic denervation similar to lack of GDNF upregulation after reinnervation of long-term denervated nerve stumps in our experiments. Loss of Schwann cell proliferation results in a progressive reduction in their number and subsequent disintegration of the endoneurial tubes that are normally maintained by the Schwann cells (Giannini and Dyck, 1990; Terenghi et al., 1998; Wood et al., 1998). Therefore, besides lack of GDNF trophic support in the long-term chronically denervated Schwann cells, other factors including the anatomical changes in the endoneurial tubes may play as significant a role as changes in the levels of neurotrophins. These long-term denervated Schwann cells maintain their capacity to switch back to the myelinating phenotype and re-myelinate regenerated axons (Wood et al., 1998; Sulaiman and Gordon, 2000), therefore the main challenges to the optimization of functional recovery after peripheral nerve injury include, at least in part, the sustenance of the levels of the upregulated regeneration-associated genes in the distal nerve stumps even after long-term chronic denervation and/or understanding the molecular mechanisms of Schwann cell response to injury and reinnervation.

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CHAPTER 4

4.0 CHRONIC SCHWANN CELL DENERVATION AND THE PRESENCE OF A SENSORY NERVE REDUCE MOTOR AXONAL REGENERATION

Adapted from the original publication:

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4.1 INTRODUCTION

Injury to the peripheral nervous system is often followed by poor functional recovery, especially after injury to large nerve trunks like the brachial or lumbar plexus (Midha, 1996). In this case, injured neurons have to regenerate their axons over a long distance, which they do at a very slow rate of 1mm/day in humans (Seddon et al., 1943). Coupled with the regenerative response in the neurons is the response of the non-neuronal Schwann cells of the distal stump of the injured nerve. Loss of axonal contact induces Schwann cell proliferation and a change in their phenotype from that of myelinating to a non-myelinating, growth-supportive phenotype. The change in phenotype is associated with down-regulation of the myelin-associated genes (e.g. P0, myelin-associated glycoprotein, MAG) and upregulation of the regeneration-associated genes (e.g. adhesion molecules, L1, neural-cell adhesion molecule, NCAM; GAP-43; for review see Scherer and Salzer,1996).

The growth-supportive environment of the distal nerve stump provided by the Schwann cells is maintained only if axonal contact is re-established. The growth-supportive environment progressively deteriorates when nerve repair is delayed or neurons have to regenerate over long distances (Fu and Gordon, 1995b). Unfortunately, the slow rate of regeneration often means that the growth supportive environment has deteriorated before the injured motoneurons are able to regenerate axons to it, reducing the amount of axonal regeneration. We have shown previously in a rat model of nerve-nerve cross-suture that, if nerve repair is delayed beyond 4 weeks, the capacity of motoneurons to regenerate axons into the distal nerve stump is compromised (Sulaiman and Gordon, 2000). Therefore, nerve repair within the first 4 weeks after injury is required for optimal functional recovery after nerve injury. Thus, ***time*** and ***distance*** are crucial factors, both of which affect the extent of functional recovery after peripheral nerve injury; these are the issues we address in this paper. In cases where immediate repair is not feasible, a popular experimental approach that has been shown to improve functional recovery after delayed repair is the temporary use of a foreign sensory nerve to neurotise the distal nerve stump ('Sensory Babysitting') prior to nerve-nerve cross suture of mixed nerves (Hynes et al., 1997; Zhang et al., 1997). This is postulated to help maintain the endoneurial pathways and/or prevent the deterioration of growth-supportive Schwann cell phenotype of the distal nerve stumps by allowing their timely reinnervation.

In the present experiments, we investigated i) the effects of delayed nerve repair (***time***) on the regeneration of motoneurons in a sensory pathway and ii) the capacity of motor or sensory neurotisation of a long sensory (***distance***) pathway to prevent poor motor axonal regeneration. We hypothesised that i) delayed nerve repair, which leaves distal sensory pathway chronically denervated, will reduce the capacity of motoneurons to regenerate in the sensory pathways and ii) that sensory or motor neurotisation of one end of a long sensory autograph will increase motor axonal regeneration from the desired motor nerve. Using retrograde neurotracing technique, we evaluated the capacity of motor neurons to regenerate axons into freshly cut and 2 month chronically denervated distal sensory nerve stumps, and their capacity to regenerate axons across a long autograft alone, or simultaneously as sensory or motor axons regenerated into the other end of the sensory autograft (sensory or motor protection of the pathway).

We found that regeneration of motoneurons into the sensory graft was reduced when the sensory distal nerve stump was chronically denervated for 2 months and when sensory axons regenerate from the other end of the long autograft. On the other hand, motor axonal regeneration was unaffected into a freshly cut distal nerve stump, or into a graft that was distally ligated and had neither sensory nor motor nerves growing from the other end of the graft or when motor nerves regenerated from the other end. These data show that both delayed nerve repair (chronic denervation) and the presence of sensory nerves in the sensory graft reduced motor axonal regeneration in the sensory pathways. Moreover, this inhibitory effect of sensory nerve axons on motor axonal regeneration was not simply as a result of occupation of the endoneurial tubes since regeneration of motor nerves did not have such an effect. These results have previously been presented in abstract forms (Midha et al., 1999; 2000).

4.2 MATERIALS AND METHODS

4.2.1 Animal Model and Groups

The motor and sensory branches of femoral nerve of female Sprague Dawley rats (Harlan Sprague Dawley, Indianapolis, IN), weighing between 250 and 300g, were used in these experiments as a model of nerve injury and repair. The rats were maintained in a standard animal facility with 12 hour on-off light conditions. They were allowed to acclimatize to the new environment prior to surgical procedures, housed in flat-bottom betachip lined cages and allowed ad libitum standard rat chow and water. All experiments and animal interventions strictly adhered to Canadian Council on Animal Care guidelines.

Rats were randomly assigned to 5 surgical groups. In groups 1 and 2, the proximal stumps of freshly cut motor nerves were sutured into either freshly cut or 2 month chronically denervated distal sensory nerve stumps, respectively (Figure 1). In group 3, the proximal stumps of the motor branch of the right femoral nerve was sutured into a saphenous nerve graft that was distally ligated (Figure 2A). In group 4, the proximal stumps of the right motor branch and the left sensory saphenous branch of the femoral nerves were sutured into the 4cm long saphenous graft harvested from the right (Figure 2B). In group 5 (Figure 2C), proximal stumps of motor branches of both right and left femoral nerves were sutured into the graft (see table 1).

4.2.2 Surgical Procedures

Anesthesia was induced in all animals by intramuscular injection of 100mg/kg ketamine hydrochloride (0.1ml/100g, Rogarsetic, Rogar-STB, Montreal, PQ) and 10mg/kg acepromazine maleate (0.1ml/100g, Atravet, Ayerst Laboratories, Montreal, PQ) into the lumbar paraspinal musculature. All surgical procedures were performed in an aseptic manner and employed standard microsurgical techniques using an optical microscope (Wild M651, Wild Leitz, Willowdale, ON). All regions that were operated on were shaved and prepared with Betadine and 70% alcohol. Nerve-nerve repairs were done using10-0 nylon (Dermalon, Davis and Geck, American Cyanamid Company, Danbury, CT) epineurial sutures.

In group 1, the right motor and sensory branches were dissected and the proximal stump of the freshly cut motor nerve was sutured into the distal stump of freshly cut distal sensory nerve, while the remaining stumps were ligated distally with 7-0 nylon. In group 2, the right sensory nerve was cut and its distal nerve stump was ligated to prevent reinnervation by the proximal stump. Two months later, following re-exposure, the proximal stump of right freshly cut motor nerve was sutured into the 2 month chronically denervated distal sensory nerve stumps.In groups 3-5, the first surgical manipulation was to harvest the right saphenous nerve. On the medial surface of the rat right hindlimb near the ankle, the saphenous nerve was isolated atraumatically from the vein, divided distally, and harvested throughout its course up to the inguinal region in order to obtain a 4cm-long graft. The graft was then subcutaneously tunneled across the lower abdominal wall to the left side of the animal. In group 3, in the right inguinal region, the proximal stump of the right motor nerve was sutured into the proximal part of the saphenous graft and the other end of the graft was ligated (motor-graft-ligation, M-G-L) using a 7-0 nylon suture. In group 4, the motor branch of the femoral nerve was isolated and cut so that its proximal stump could be sutured to the proximal part of the

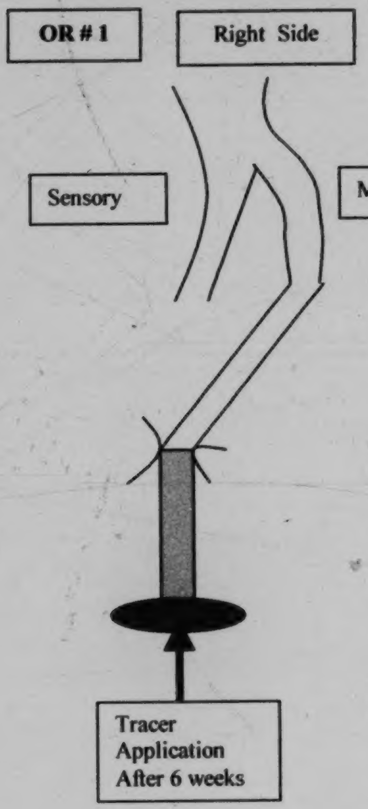
Table 4.1: Animal groups and surgical procedures (R-right; L-left)

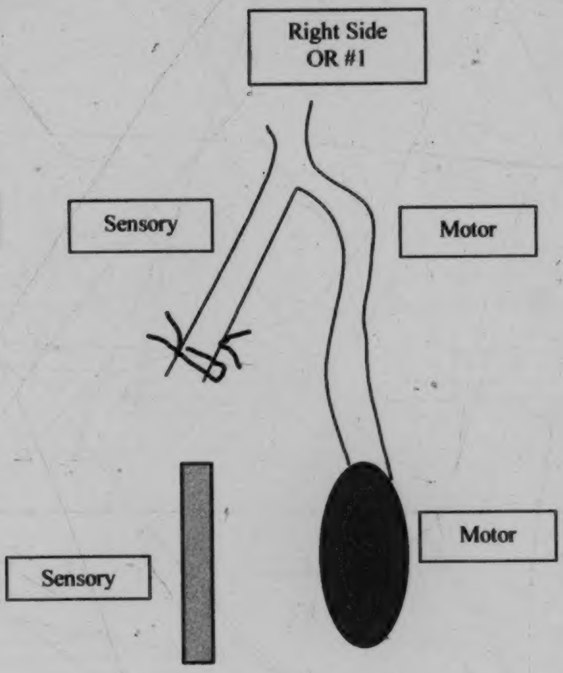
Group	Surgery	Abbreviation	N
1	R motor sutured to freshly cut R distal sensory nerve stump	M-S	8
2	R motor sutured to 2 month chronically denervated R distal sensory nerve stump	M-dS	8
3	R motor sutured to distally ligated saphenous graft	M-G-L	7
4	R motor and L sensory sutured to saphenous graft	M-G-S	6
5	R motor and L motor sutured to saphenous graft	M-G-M	4

Figure 4.1: Schematic diagram of the motor-sensory nerve cross-suture to examine the effect of 2 month sensory distal nerve stump chronic denervation on motor axonal regeneration. The sensory saphenous nerve branch of rat femoral nerve was cut and the proximal stump of freshly cut quadriceps motor nerve branch was immediately sutured to the distal stump of the cut sensory nerve (group 1, OR#1). Regeneration of the femoral motoneurons was allowed for 6 weeks after which the number of femoral motoneurons that regenerated their axons into the sensory distal nerve stump was estimated by application of either fluorogold (FG) or fluororuby (FR) to the cut end of the sensory nerve. In group 2, the sensory nerve was cut, the proximal and distal stumps were separated and ligated to subject the distal stump to chronic denervation (OR #1). Two months later (OR#2), motor-sensory cross-suture was performed as in group1 to allow regeneration of motor axons into the distal sensory nerve stump. Again, after 6 weeks, FG or FR was applied to identify and count the number of femoral motoneurons that regenerated axons into the 2 month chronically denervated distal sensory nerve stump.

REPAIR FOLLOWING PROLONGED DISTAL STUMP DENERVATION

Group 1 (Immediate repair)


OR # 1
Right Side
Sensory
Motor
Tracer Application After 6 weeks


Right Side OR #1
Sensory
Motor
Motor
Sensory

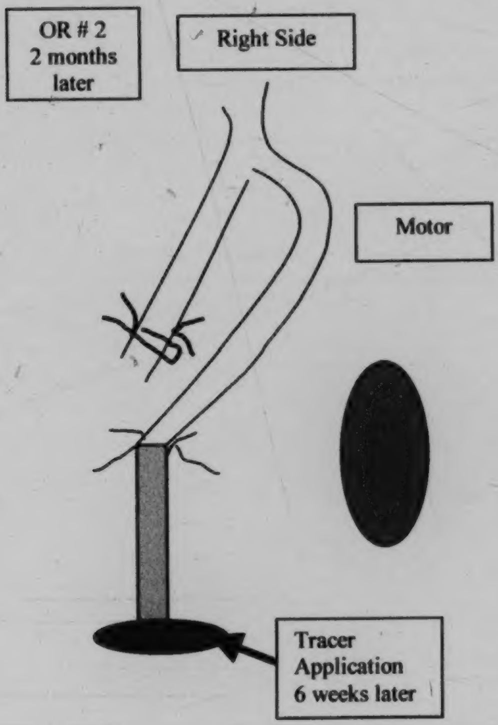
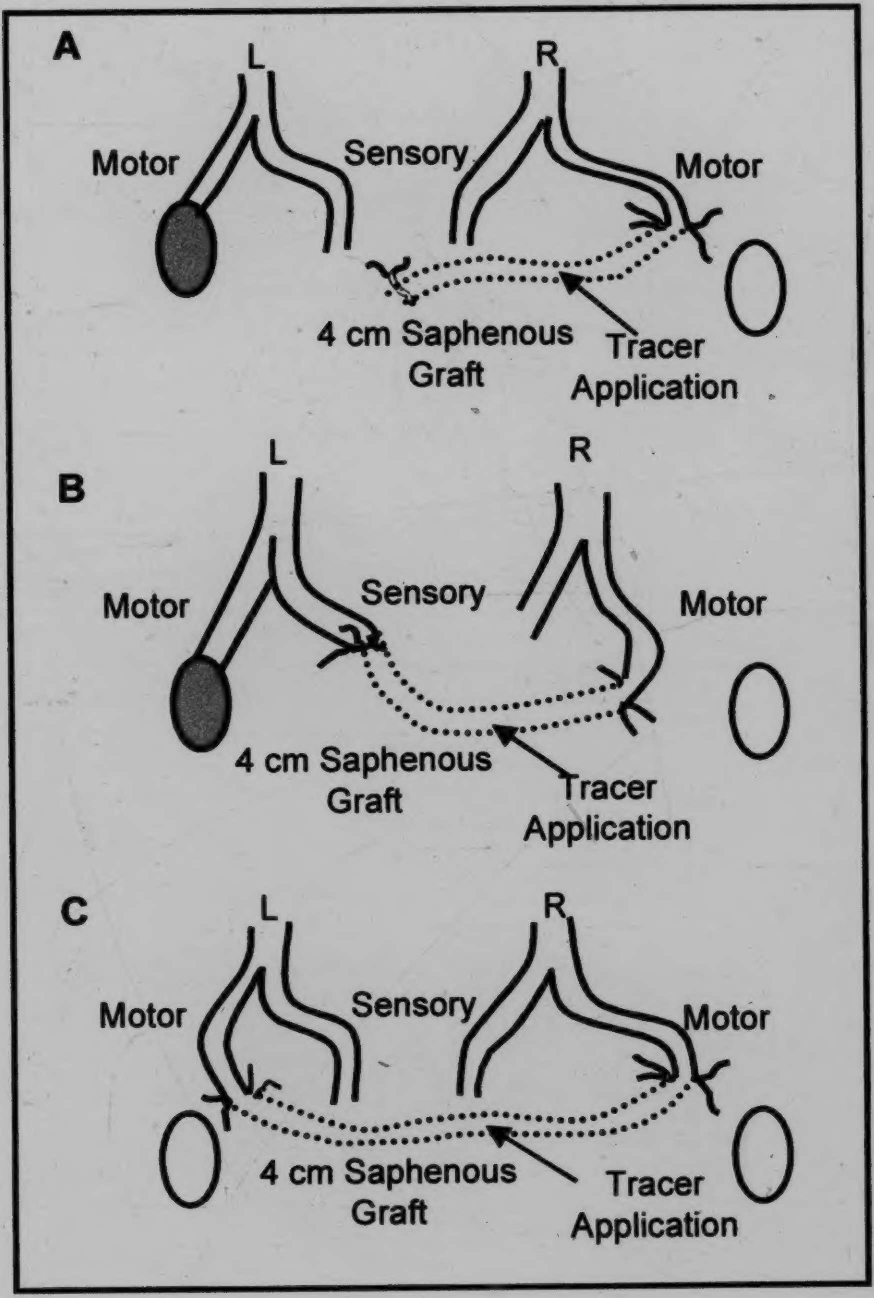

OR # 2 2 months later
Right Side
Motor
Tracer Application 6 weeks later

Figure 4.2: Schematic representation of motor axonal regeneration into sensory nerve graft alone or in the presence of sensory or motor nerves (groups 3-5). In the 3 groups of rats, the first surgical manipulation was to harvest the right saphenous nerve. The graft was then subcutaneously tunneled across the lower abdominal wall to the left of the animals and the surgical repairs were concluded in the 3 groups as follows: A) In group 3, in the right inguinal region, the motor branch of the femoral nerve was isolated and cut so that its proximal stump could be sutured to the proximal part of the saphenous graft. The other end of the graft was ligated (motor-graft-ligation, MGL); B) In group 4, after suturing the proximal motor nerve to the graft on the right side, the other end of the graft was sutured to the proximal stump of the freshly cut left saphenous nerve in the inguinal region (motor-graft-sensory, MGS); C) in group 5, both the right and left proximal motor nerves were sutured to the saphenous graft on the right and left respectively (motor-graft-motor, MGM).


A
L
R
Motor
Sensory
Motor
4 cm Saphenous Graft
Tracer Application
B
L
R
Motor
Sensory
Motor
4 cm Saphenous Graft
Tracer Application
C
L
R
Motor
Sensory
Motor
4 cm Saphenous Graft
Tracer Application

saphenous graft (Figure 2B) and its distal stump was reflected distally and ligated with a 7-0 nylon suture. The other end of the graft was sutured to the proximal stump of the left saphenous sensory nerve in the inguinal region after it was cut; the distal stump of the left saphenous nerve was also ligated distally with a 7-0 nylon suture (Figure 2B; motor-graft-sensory, M-G-S). Group 5 had a similar right motor to graft repair, but the distal part of the saphenous graft was sutured to the proximal stump of the left motor branch of the femoral nerve instead of the sensory saphenous nerve branch (Figure 2C; motor-graft-motor, M-G-M).

The skin incisions were approximated with continuous 3-0 nylon suture and cleaned, and animals were allowed to recover. Regeneration into the nerve graft from both sides (groups 4 and 5) or from one side (group3) was allowed for 3 months. Regeneration into both freshly cut or chronically denervated sensory nerve stumps (groups 1 and 2) was allowed only for 6 weeks, the time required for all femoral motoneurons to traverse the required distance of 20mm (Al-Majed et al., 2000). At the end of the regeneration times, assessment of motor regeneration in the different animal groups was carried out using retrograde neuroanatomical tracing technique.

4.2.3 Retrograde Labeling of Regenerated Motoneurons

In order to determine the number of motoneurons that regenerated axons into the saphenous nerve grafts and distal sensory nerve, retrogradely transported axonal tracers were used to backlabel femoral motoneurons. Fluorogold (FG; Fluorochrome Inc. Denver) and Fluororuby (RF; Dextran tetramethylrhodamine, Mol. Probes, D-1817, Eugene, OR) were the 2 dyes chosen since they are effectively endocytosed and retrogradely transported (Richmond et al., 1994; Novikova et al., 1997). In rats in groups 3-5, a small incision was made in the abdomen to expose the graft, which was sectioned in the centre (approximately 2cm from the nerve repair sites on either sides). A 3mm long piece of reinnervated saphenous graft was dissected and excised from both sides of the sectioned graft for light microscopy evaluation (see below). Thereafter, the tip of each part of the graft was exposed to either FG or FR. The fluorescent dye used was alternated between animals to control for possible differences in retrograde uptake and transport of the dyes. Backlabeling with FG was done by exposing the tip of the cut saphenous nerve graft to 4% FG in 0.1 M cacodylic acid for 1 hr (Schmued and Heimer, 1990) in a vaseline well, after which it was extensively irrigated and reflected to a distant portion of the wound. Backlabeling with FR was done by placing the tip of the cut saphenous nerve graft on a 1cm^2 weighing paper (Fisher Scientific Co., USA) with FR crystals for 2 h, then irrigating the nerve and placing it in the opposite corner of the wound to prevent backlabeling of non-femoral motoneurons by diffusion of tracers. After exposing the motor-sensory suture site in the inguinal region, similar procedure was used for backlabeling motoneurons which regenerated their axons into freshly cut and 2-month chronically denervated sensory distal nerve stumps (groups 1and 2). Animals were again allowed to recover and maintained for 6 days to allow for retrograde transport of the applied fluorescent dyes to the neuronal cell bodies.

4.2.4 Tissue Perfusion and Nerve Histology

On the 5th day after the application of the retrograde dyes, rats were deeply anaesthetized with sodium phenobarbital (30mg/kg i.p.) and perfused with 100ml of saline followed by 500 ml of ice cold 4% paraformaldehyde (pH 7.4) through the aorta. After perfusion, the part of the thoracolumbar spinal cord which contains all the femoral motoneurons (T11-L2) was removed and post fixed in 30% sucrose in 4% paraformaldehyde solution overnight. Tissues were then frozen in liquid nitrogen and stored at -80°C prior to cryostat sectioning.

The nerve pieces that were taken out before application of fluorescent dyes were fixed in gluteraldehyde (3% in 0.1M phosphate buffer), stained with osmium tetroxide (3% solution in 0.1M phosphate buffer), dehydrated in ascending alcohols and embedded in araldite. Sections of 1.5um thickness were stained with methylene blue for photomicrographs. The total number of regenerated fibres were enumerated by saving low magnification (40X) images of the whole nerve section to the JAVA system (Jandel Scientific, USA). All regenerated fibres (myelinated and unmyelinated) in each image were counted using the JAVA imaging system. Fibre diameter, myelin thickness were also determined using this system.

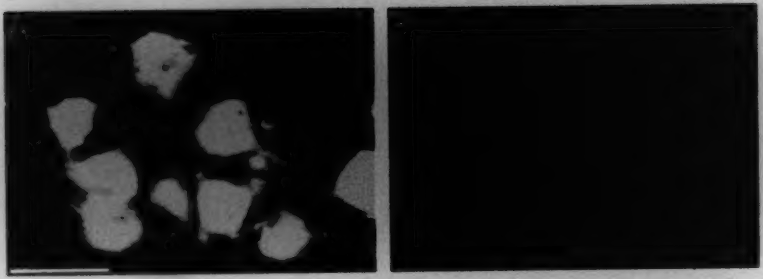
4.2.5 Motoneuron Counting

Each thoracolumbar spinal cord was mounted horizontally on a cryostat chuck with the dorsal part of the spinal cord facing up for sectioning of 50μm longitudinal sections using a freezing microtome (Jung CM 3000). Sections were serially mounted on glass slides, allowed to dry and coverslipped. Retrogradely labeled motoneurons in each spinal cord section were observed and all the motoneurons counted at 20-40X magnification under UV fluorescence at barrier filters of 430nM for FG and 580nM for FR. Cells labeled with FR could be easily identified by their red fluorescence and those labeled with FG had blue fluorescence (Figure 3). Backlabeled femoral motoneurons which regenerated their axons into the saphenous nerve graft or into the sensory distal nerve stumps were identified and enumerated. Only the motoneurons in which there was a distinct nucleus and that could be visualized only under the UV fluorescent filter specific for the dye with which they were labeled were counted; multiple counting of split neurons was corrected for by the method of Abercrombie and Johnson (1946).

4.2.6 Statistical Analysis

One-way analysis of variance (ANOVA) and the Student's *t* test were used to compare the mean number of femoral motoneurons that regenerated axons and the respective number of axons in all groups of rats. Statistical significance was accepted at the 5% level ($p<0.05$).

Figure 4.3: Photomicrographs of motoneurons backlabelled with FG (A) and FR (B) as viewed under the fluorescent microscope. Both FG and FR labeled the soma and dendrites of femoral motoneurons that regenerated axons in the sensory pathways. FG-labeled motoneurons were identified by their blue fluorescence and those labeled with FR by their red fluorescence. The scale bar is 30um.



4.3 RESULTS

4.3.1 Femoral Motoneuron Regeneration into Distal Sensory Nerve Stump Was Reduced by Chronic Denervation of the Distal Sensory Nerve Stump

Consistent with previous report by Al-Majed et al., (2000), a total of 315 ± 24 femoral motoneurons, which represent all femoral motoneuron pool, regenerated their axons (as per number of FG or FR-backlabeled femoral motoneurons) 20mm into distal sensory nerve stump after immediate coaptation of freshly cut proximal motor and distal sensory nerve stumps and a regeneration period of 6 weeks. However, delayed nerve repair which subjected the distal sensory nerve stump to 2 month chronic denervation, reduced the number of femoral motoneurons that regenerated from a total of 315 ± 24 (after immediate coaptation) to 254 ± 20 (Figure 4A). This data demonstrate a similar effect of chronic denervation of regeneration of freshly cut tibial nerve stump into chronically denervated distal common peroneal nerve stump (Sulaiman and Gordon, 2000). Thus, chronic denervation has the same effect on motor axonal regeneration regardless whether motor nerves regenerate into mixed or sensory nerves.

4.3.2 Regeneration of Femoral Motor Axons into Saphenous Graft in the Absence of Both Motor or Sensory Nerve

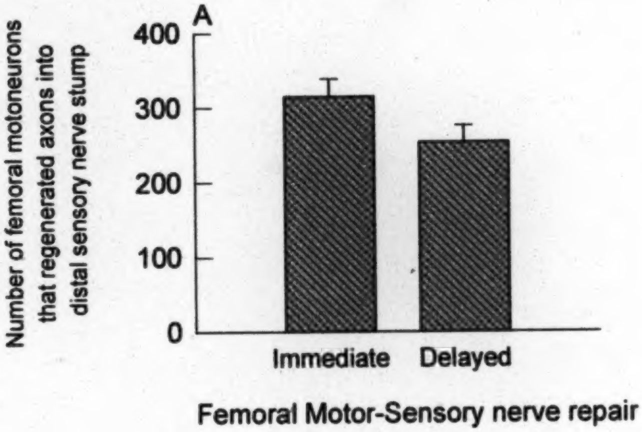
We used retrograde fluorescent labeling to identify and count the number of right femoral motor neurons that regenerated their axons 2 cm into a 4cm-long saphenous graft that was ligated at the other end, 3 months after motor-saphenous graft repair (group 3). Both FG and FR labeled all regenerated femoral motoneurons (figure 3) and there was no significant difference between the numbers of backlabeled motoneurons using either FG or FR. Femoral motoneurons regenerated into

the saphenous nerve graft, consistent with previous observation of regeneration of motor axons in sensory pathways (Figure 4.4A; Kilvington, 1941). A mean (± SE) of 151 ± 5 motor neurons regenerated 2cm into the saphenous graft (figure 4B, MGL). Counting of the total number of regenerated axons in the nerve graft showed that a total of 1522 ± 81 axons regenerated, which included both sensory and motor fibers (figure 4C, MGL). Light and electron microscopic evaluation demonstrate multiple sprouts consistent with previous observation when axons regenerated into blind ends (Weiss et al., 1945; figure 5, MGL) or ligated nerve stumps (Midha et al., 1998). Hence, the high number of regenerated axons is accounted for partly by the multiple axonal sprouts. Because, the axons are mostly sprouts, the nerve fiber diameters are significantly smaller compared to when axonal regeneration is not frustrated by a distal ligature ($p<0.05$; figure 6A, MGL).

4.3.3 Regeneration of Femoral Motor Axons into Saphenous Graft in the Presence of Regenerating Sensory Axons

Right femoral motoneurons regenerated into a 4cm-long saphenous graft while sensory axons regenerated from the other end of the graft on the left. Both sensory and motor neurons were allowed to regenerate for a period of 3 months prior to application of fluorescent dyes to backlabel and count the number of femoral motor neurons that regenerated axons 2 cm into a 4cm-long saphenous graft in the presence of regenerating sensory nerve. In the presence of regenerating sensory nerve, significantly fewer ($p<0.05$) femoral motor axons regenerated into the saphenous graft, 99 ± 2 motor neurons, compared to 151 ± 5 motor neurons that regenerated in the absence of sensory nerve (figure 4B, MGS). Hence, the presence of regenerating sensory nerve in a sensory nerve graft reduced the number of motoneurons that regenerated simultaneously in the same sensory pathways from the opposite side. The total number of regenerated axons was also reduced from 1522 ± 81 when motor nerve regenerated alone to 516 ± 44 in the presence of sensory nerve (figure 4C, MGS). Light and electron microscopic evaluation showed significant evidence of myelin and axonal degeneration and the presence of large axons that were non- or thinly-myelinated (figure 5, MGS; figure 6D). The large size of the regenerated axons and the lack of adequate myelination is reflected in the plots of axon diameters and axon/myelin ratios (Figures 6C and 6D). The frequency histograms (figure 7) clearly demonstrate that more large axons regenerated in the MGS groups, as shown by the highest frequency at 3um and the presence of more nerve fibers which are 4-5um in diameter. The axonal/myelin degeneration and the presence of some large thinly myelinated or unmyelinated axons resemble the pathological features seen following withdrawal of cyclosporin immunosuppression after the use of nerve allograft, especially when motor nerves regenerate through the allografts into sensory pathway (Midha et al, 1997). This suggests that the presence of sensory axons in a sensory pathway reduces the interaction of sensory Schwann cells with regenerated axons, presumably motor, as far as myelination and axonal integrity are concerned.

Figure 4.4: The numbers of femoral motoneurons that were Backlabeled with either FG or RR in the spinal cord were counted and plotted (mean ± SE) as a function of A) immediate or delayed motor-sensory cross nerve repair, B) femoral motoneuron regeneration in the sensory nerve graft alone (MGL, filled black bars), in the presence of sensory (MGS, filled grey bars) or motor (MGM, white stripped bars) nerves. The graph in C illustrates the corresponding changes in the numbers of regenerated nerve fibers in the MGL, MGS and MGM groups. Two month chronic denervation of distal sensory nerve stump and the presence of sensory nerve (MGS group) significantly reduced the number of femoral motoneurons that regenerated axons into the distal sensory nerve stumps ($p<0.05$, ANOVA and Students' t test) and the number of regenerated nerve fibers in the MGS group (C).


A
Number of femoral motoneurons that regenerated axons into distal sensory nerve stump
400
300
200
100
0
Immediate
Delayed
Femoral Motor-Sensory nerve repair

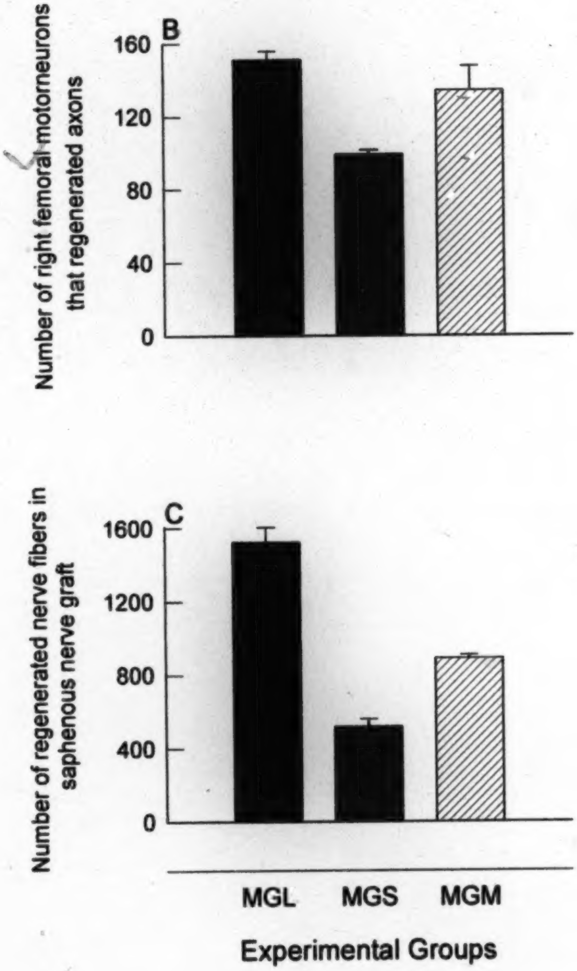
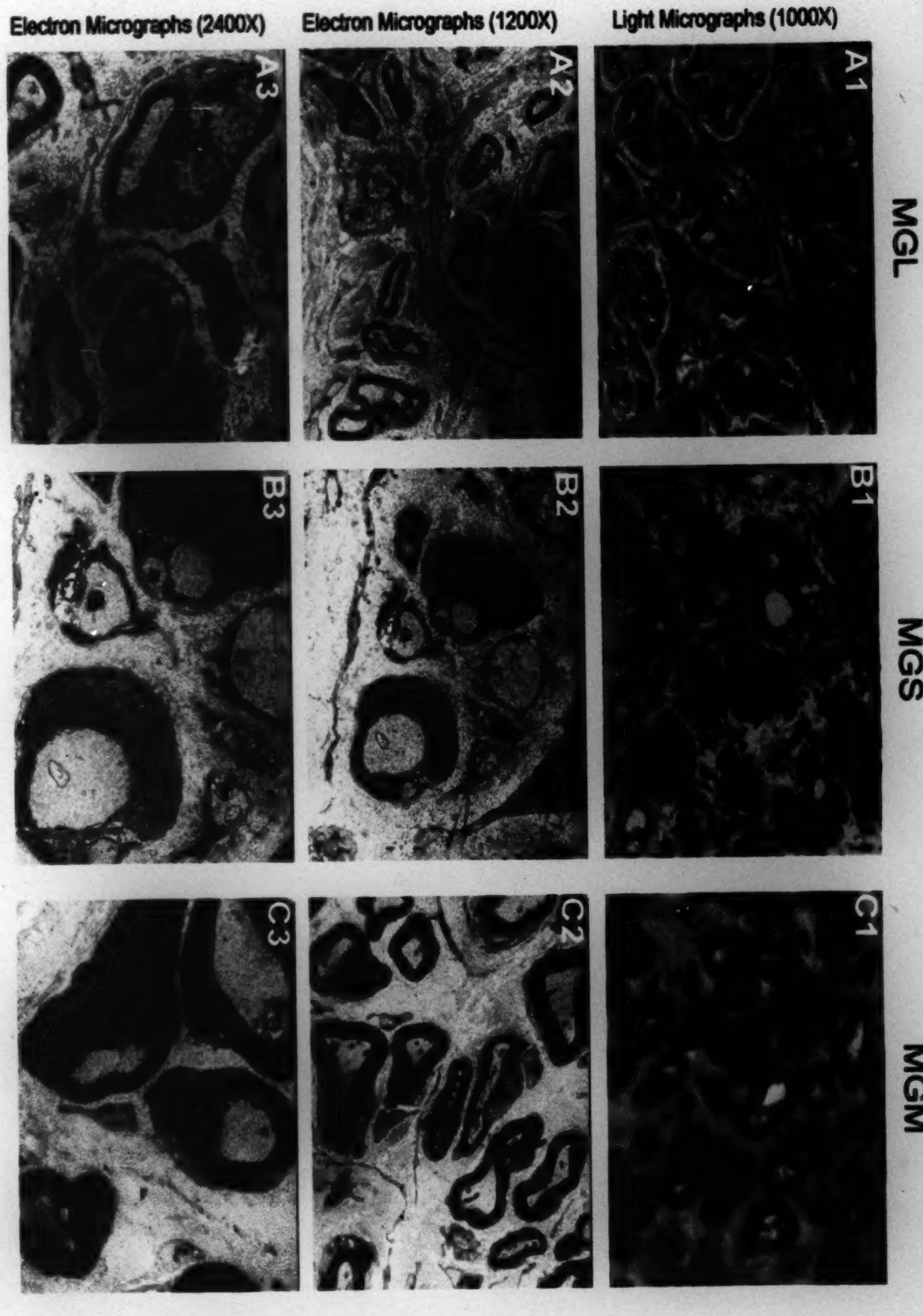

B
Number of right femoral motorneurons that regenerated axons
160
120
80
40
0
C
Number of regenerated nerve fibers in saphenous nerve graft
1600
1200
800
400
0
MGL
MGS
MGM
Experimental Groups

Figure 4.5: Light (A1, B1, C1) and electron micrographs (A2,A3;B2,B3;C2,C3;) of regenerated nerve fibers from the quadriceps motor branch of the femoral nerve. Axons which regenerated into a saphenous nerve graft that was ligated at the other end (MGL) formed multiple sprouts and were well myelinated. Note the predominance of regenerating units in (A1-A3).The presence of sensory nerve (MGS) reduced the responsiveness of sensory Schwann cells to myelinate regenerated motor axons as demonstrated by multiple large axons with little or no myelin (B1-B3).In the presence of motor nerves (MGM), regenerated axons are larger and well myelinated by the sensory Schwann cells (C1-C3). Magnifications are indicated on the micrographs.


Electron Micrographs (2400X)
Electron Micrographs (1200X)
Light Micrographs (1000X)
A3
A2
A1
MGL
B3
B2
B1
MGS
C3
C2
C1
MGM

4.3.4 Regeneration of Femoral Motor Axons into Saphenous Graft in the Presence of Regenerating Motor Axons

Both right and left femoral motoneurons regenerated concurrently into a 4cm-long saphenous graft for a period of 3 months. Fluorescent dyes were then used to backlabel all motor neurons that regenerated axons 2cm into the saphenous nerve graft from both the right and left sides. We found that 134 ± 13 (mean ± SE) motor neurons regenerated into the saphenous graft from the right motor nerve, almost identical to the 135 ± 9 motoneurons that regenerated from the left. These numbers are also similar to the number of motor neurons (151 ± 5) that regenerated into the saphenous graft without sensory or motor nerves regenerating from the other end of the graft (figure 4B, MGL and MGM). Also, the number of regenerated axons was not as reduced as compared to motor axons regenerated in the presence of sensory axons, although it was significantly less than the number of axons when motor nerve regenerated alone (from 1522 ± 81 to 888 ± 18). This significant difference could be attributed to the multiple sprouts observed when motor nerves regenerated into ligated nerve graft. All regenerated axons were well myelinated (figure 5, MGM).

The lack of reduction in the number of femoral motoneurons that regenerated axons indicates that the reduction in the number of regenerated motor axons in the presence of sensory nerve was not simply due to competition for a finite number of endoneurial tubes. Rather, there could be an inhibitory influence from the presence of sensory axons on motoneuronal regeneration, which may be from axonal or Schwann cell-derived factors.

Figure 4.6: Histograms depicting the changes in regenerated nerve parameters when motor nerves regenerated alone (MGL, filled black bars), in the presence of sensory nerve (MGS, filled grey bars) or motor nerve (MGM, stripped white bars). A and B: the presence of motor nerve increased both diameter and myelination of regenerated motor nerve fibers in the saphenous nerve graft (MGM) whereas the presence of sensory nerve reduced both parameters (MGS); C and D: Despite the large size of most regenerated axons in the presence of the sensory nerve, they were only poorly myelinated (MGS), as reflected in the substantially increased axon/myelin ratio.

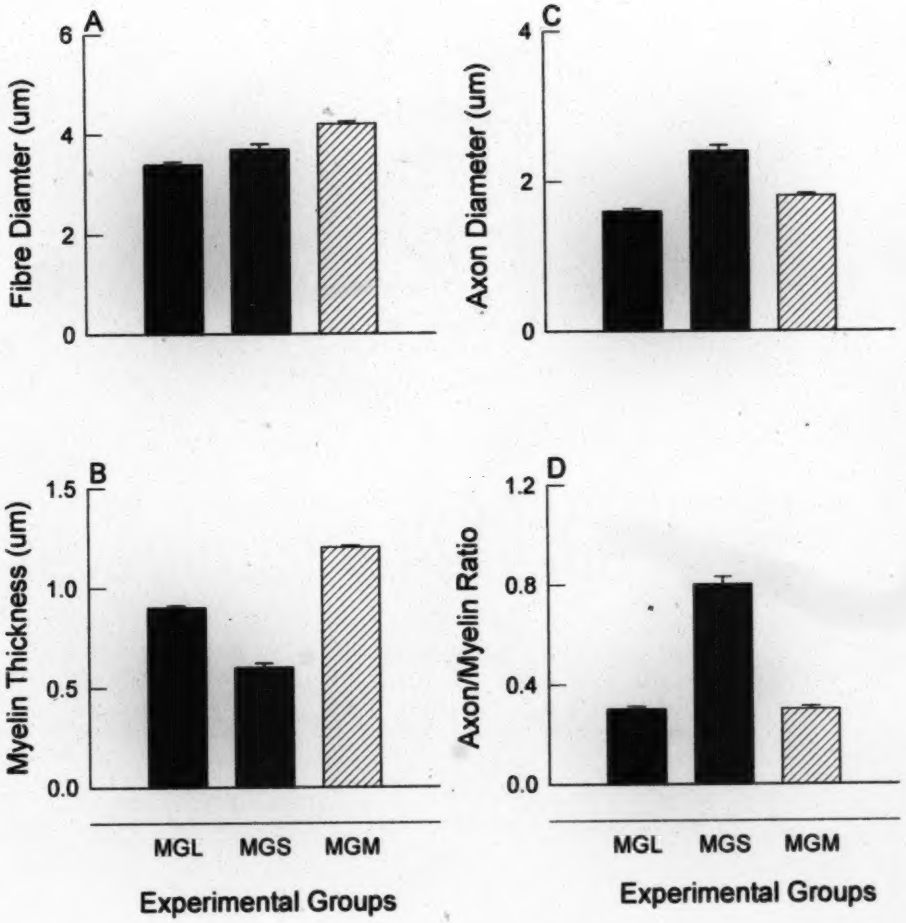
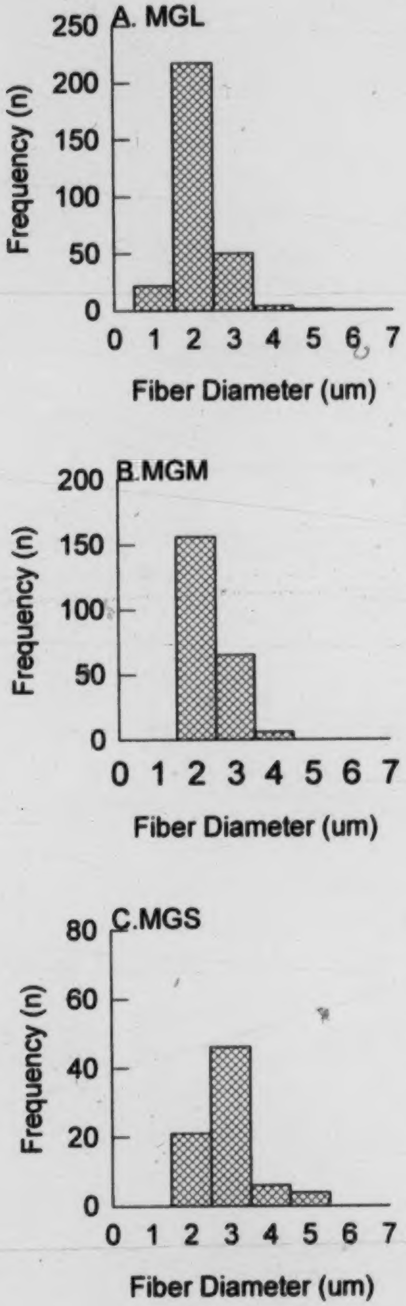

A
Fibre Diamter (um)
6
4
2
0
C
Axon Diameter (um)
4
2
0
B
Myelin Thickness (um)
1.5
1.0
0.5
0.0
D
Axon/Myelin Ratio
1.2
0.8
0.4
0.0
MGL
MGS
MGM
Experimental Groups
MGL
MGS
MGM
Experimental Groups

Figure 4.7: Frequency histograms of regenerated nerve fibers in the MGL (A), MGS (B) and MGM (C) groups. Note that nerve fibers which regenerated in the presence of motor nerves (C, MGM) have the same frequency distribution of nerve fiber diameters as in the MGL group (A) whereas the presence of sensory nerves shifted the peak frequency of fiber diameter from 2um in the MGL and MGM groups to 3um, and there are more nerve fibers with diameters in the 4-5um range (B).


A. MGL
Frequency (n)
250
200
150
100
50
0
0 1 2 3 4 5 6 7
Fiber Diameter (um)
B.MGM
Frequency (n)
200
150
100
50
0
0 1 2 3 4 5 6 7
Fiber Diameter (um)
C.MGS
Frequency (n)
80
60
40
20
0
0 1 2 3 4 5 6 7
Fiber Diameter (um)

4.4 DISCUSSION

4.4.1 Effect of Chronic Denervation on Motor Axonal Regeneration

Schwann cells in the denervated distal nerve stumps of injured nerves are essential for successful axonal regeneration (Hall, 1986a,b). However, the capacity of Schwann cells to maintain their growth-supportive capacity is dependent on timely reinnervation by regenerated axons. Timely reinnervation is denied in the majority of cases because of the slow rate of regeneration, a situation that is magnified when the regeneration must proceed over long distance. This problem is aggravated by delayed nerve repair. We have recently shown in a rat model of nerve-nerve cross-suture that, if nerve repair is delayed beyond 4 weeks, the capacity of motoneurons to regenerate axons into the distal nerve stump is compromised (Sulaiman and Gordon, 2000). In this recent study, we found that it is the progressive failure of the distal nerve stumps rather than failure of chronically denervated muscles to accept innervation that accounts for the progressive failure of axonal regeneration with time and distance. In the present study, immediate motor-sensory repair results in regeneration of all femoral motoneurons into the sensory pathways. However, when motor-sensory nerve repair is delayed for 2 months, the capacity of femoral motoneurons to regenerate axons was reduced by almost 20%, similar to the effect of chronic denervation on tibial nerve regeneration. Hence, regardless of nerve types, timely reinnervation of the injured distal nerve stumps is essential for optimal axonal regeneration.

The effect of chronic denervation on axonal regeneration could be explained by the time-related changes in the Schwann cells of the distal nerve stumps. Following nerve injury, loss of axonal contact initiates dramatic changes in Schwann cells which include upregulation of a multitude of regeneration-promoting factors such as growth factors and adhesion molecules (for review see Fu and Gordon, 1997). However, the up-regulation of regeneration-associated gene expression is not maintained indefinitely, but often is very transient and finite (Carraway and Burden, 1995; Scherer and Salzer, 1996; Carroll et al., 1997; You et al., 1997). Therefore, a time window (probably 0-4 weeks) seems to exist during which the Schwann cell environment in the distal nerve stump is most supportive of axonal growth and muscle reinnervation. Beyond this period, axonal regeneration is reduced, consistent with what we observed when motor reinnervation of distal sensory nerve is delayed for 2 months (figure 4A).

4.4.2 Axonal Regeneration into a Ligated Sensory Nerve Graft

Parent axons in the proximal stump of injured peripheral nerve elaborate multiple growth cone and produce many daughter axons which cross the repair site and reinnervate the distal nerve stump (Shawe, 1955; Morris et al., 1972). A fewer number of daughter axons continue their growth along the distal nerve stump as they compete for a limited number of Schwann cell-lined endoneurial pathways in the distal nerve stump (Gutmann and Sanders, 1943; Bunge, 1987). If the growth of the axons that continue to traverse the distal nerve stump is impeded, as it is in our experiments when motor axons regenerated into a nerve graft that is ligated at the opposite end (MGL), these axons elaborate multiple sprouts again (figure 5, MGL). This phenomenon was first illustrated by Weiss et al., (1945) in which they showed a threefold increase in fiber number when nerve regeneration was impeded. The mechanism of the formation of multiple sprouts is very similar to the increased branching of fibers proximal to the area of constriction in suture-ligature created neuromas (Fried et al., 1991). The axons that regenerated into the ligated end of the sensory grafts are denied assess to end organs and therefore may not undergo the usual pruning and resorption that axons which are inappropriately connected normally undergo (Brushart, 1988, 1991, 1993). Thus, the combination of increased sprouting of regenerated axons proximal to the ligature of the sensory graft and decreased resorption of these axons probably account for the significantly greater nerve fiber counts in the MGL rats.

4.4.3 Axonal Regeneration in Sensory Graft in the Presence of Sensory and Motor Axons

The number of motoneurons which regenerated axons into sensory pathways was significantly reduced when sensory axons regenerated from the other end in the same pathways (Figure 4B). The number and myelination of regenerated nerve fibers were also significantly reduced in the presence of sensory axons (figure 4C; figure 5, MGS). Therefore, one of the most intriguing results from the present study is that the presence of sensory nerve axons decreased motoneuron regeneration and myelination. We speculate that this effect may represent a reduced or poor interaction between regenerated axons and Schwann cells occupied by sensory axons, which in turn, somehow impaired the capacity of "sensory" Schwann cells to support motor axonal regeneration and remyelination. This observation is not due to a mere occupation of the endoneurial pathways but rather appears to be an active inhibition by the sensory nerve as the reduction in regeneration is not observed when motor nerve regenerated from the other end of the graft. On the contrary, the presence of motor nerve improved the size and myelination of regenerated nerve fibers (figure 6A-D). A similar inhibitory influence of the presence of sensory axons on motor axonal regeneration into nerve allografts and their persistence after cyclosporin withdrawal has been reported (Midha et al., 1997). Specifically, both the nerve fiber density and myelinaton were significantly reduced when motor axons, which regenerated through the allografts, were sutured to sensory distal nerve stump as compared to when the distal nerve stump was motor (Midha et al., 1997). More recently, it has been demonstrated that motor axons in mixed nerve which regenerated into nerve allografts survive better after cyclosporin withdrawal if the sensory component of the mixed nerve was eliminated by ablation of dorsal root ganglion neurons (Midha et al, 2001). Therefore, the negative effect of sensory nerves on motor axonal regeneration and survival is fairly well established although the exact mechanism is not understood. In our experiments, it seems that the sensory axons make the Schwann cells of the sensory autografts (pathway) less supportive of regenerated motor axons as far as their integrity, survival and myelination are concerned.

4.4.4 The 'Babysitting technique' and the Negative Effect of Sensory Nerve on Motor Axonal Regeneration

Chronic denervation of the distal nerve stumps beyond 4 weeks prior to nerve repair compromises motor axonal regeneration (Sulaiman and Gordon, 2000). Therefore, nerve repair within the first 4 weeks after injury is required for optimal functional recovery after nerve injury. In cases where immediate repair is not feasible, a popular experimental approach that has been shown to improve functional recovery after delayed repair is a temporary use of a foreign sensory nerve to neurotise the distal nerve stump ('Sensory Babysitting') prior to nerve-nerve cross suture of mixed nerves (Hynes et al., 1997; Zhang et al., 1997). Proponents of this procedure explain the positive effect of this approach as a trophic effect of the sensory nerve on the denervated muscles to prevent their atrophy. This argument stems from the dogma that poor functional recovery is due to the inability of denervated muscles to recover from denervation atrophy and accept reinnervation, hence functional improvement from these experiments were automatically attributed to improved muscle viability and recovery (Hynes et al., 1997; Zhang et al., 1997). However, we have previously demonstrated conclusively, using retrograde labeling to identify and count the number of motoneurons that regenerated their axons into the distal nerve stumps as a function of duration of chronic denervation of the nerve stumps, that it is the progressive failure of motoneurons to regenerate their axons into the distal nerve stumps as chronic denervation is prolonged beyond 4 weeks that accounts for the poor muscle reinnervation and recovery (Sulaiman and Gordon, 2000). This reduction in the number of motoneurons which regenerated axons in the distal nerve stumps paralleled the number of reinnervated motor units, hence motoneurons which succeed in regenerating their axons into the distal nerve stumps, reinnervated the denervated muscles regardless of duration of chronic denervation. Moreover, regenerated motoneurons were able to enlarge their motor unit size, which strongly supports the sustained viability of denervated muscles and their receptiveness to regenerated axons (Fu and Gordon, 1995b; Sulaiman and Gordon, 2000) contrary to the previously held concept that denervated muscles cannot recover from denervation atrophy and accept reinnervation. Hence, chronic denervation of the distal nerve stumps plays a primary and major role in determining the functional outcome after nerve injury, and the role of denervated muscle is secondary.

Interestingly, the 4 week period during which optimal regeneration was observed coincides with period of active Wallerian degeneration and macrophage infiltration of the distal nerve stumps, during which regenerated-associated molecules are highly expressed (Liu et al. 1995; You et al., 1997). This provides further empirical evidence that the cellular and molecular events that are induced during Wallerian degeneration are essential for optimal axonal regeneration. Therefore, it is more likely that the positive effect of sensory neurotisation is due to either and/or both i) the preservation of the distal nerve stump by immediate reinnervation by the sensory axons which induces Schwann cell proliferation (Pellegrino and Spencer, 1985) and their expression of neurotrophins, such as glial-derived neurotrophic factor (unpublished results), ii) reinduction of the trophic process of Wallerian degenration when the sensory nerve is cut and the mixed nerve is sutured to the babysat distal nerve stump.

In addition, the present study demonstrates that the presence of sensory nerve axons did not improve motor axonal regeneration and myelination but rather reduced both parameters, which suggests that the sensory nerve may be inducing a phenotype in Schwann cells that make them less responsive to regenerating motor nerves. For example, it may reduce the expression of L2/HNK-1 by Schwann cells, a molecule that is essential for the interaction of the Schwann cells with motor axons (Martini et al., 1994) or may induce some other molecule that are repulsive to motor axons. On the other hand, the positive effect of motor nerve on motor regeneration may be associated with the specific induction of molecules such as L2-HNK1. Hence, distal nerve stumps that have been previously neurotised by sensory nerves may need to undergo Wallerian degeneration prior to motor reinnervation, in order to achieve a positive effect of sensory neurotisation. Wallerian degeneration, as mentioned above, is accompanied by a change in Schwann cell phenotype and upregulation of growth-supportive molecules in the distal nerve stumps and denervated muscles (for review see Fu and Gordon, 1997), which may account for the positive effect of sensory neurotisation, although based on the evidence from this study, neurotisation by motor nerve prior to motor reinnervation may have a more pronounced positive effect than sensory neurotisation. Experiments which look at the effects of sensory neurotisation on axonal regeneration in the absence of Wallerian degeneration and comparisons of changes in gene expression in the distal nerve stumps with or without sensory would further elucidate the exact mechanism of sensory neurotisation.

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CHAPTER 5

5.0 FK506 INCREASES PERIPHERAL NERVE REGENERATION AFTER CHRONIC AXOTOMY BUT NOT AFTER CHRONIC SCHWANN CELL DENERVATION

Adapted from the original publication:

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5.1 INTRODUCTION

Despite the capacity of Schwann cells of the peripheral nervous system to support axonal regeneration, clinical experience has shown that functional recovery is often poor even after microsurgical repair. This is especially true for injuries that sever large nerve trunks or after delayed nerve repair. After injuries to large nerve trunks, motoneurons are required to regenerate over long distance, which they do at a very slow rate (Fu and Gordon, 1997). We have shown previously that the long delays incurred by the slow rate of regeneration of injured neurons over the required distances between the nerve lesion sites and the denervated targets, 1) detrimentally affect the regenerative capacity of motoneurons which remain without targets during a prolonged period of regeneration (*chronic axotomy*; Fu and Gordon, 1995a), 2) lead to deterioration of the growth-permissive Schwann cell environment of the distal nerve stumps *(chronic denervation;* Fu and Gordon, 1995b; Sulaiman and Gordon, 2000). Thus, injured motoneurons remain chronically axotomised and the Schwann cells of the distal nerve stumps remain chronically denervated as a result of their delayed reinnervation. Likewise, delayed nerve repair leads to chronic motoneuron axotomy and chronic Schwann cell denervation. Therefore, slow rate of axonal regeneration constitutes a major deterrent to functional recovery after peripheral nerve injury.

The immunphilin ligand FK506 is a potent immunosuppressant used extensively in preventing rejection after organ transplantation (Starzl et al., 1987, 1989; Hoffmann et al., 1990), an effect mediated via binding to the FK506 binding protein 12 (FKBP-12). Binding of FK506 to the FKBP-12 results in the formation of the FK-506/FKBP-12 complex which inhibits T-cell proliferation through its inhibition of calcium and calmodulin-dependent protein phosphatase calcineurin (Liu et al., 1991; Clipstone and Crabtree, 1993; Wiederrecht et al., 1993). This prevents the dephosphorylation of NF-AT by calcineurin required for activation of IL-2 gene transcription. Suppression of IL-2 production is followed by reduction of T-cell proliferation and immunosuppression.

In addition to its immunosuppressive effects, FK506 has also been shown to enhance neurite outgrowth *in vitro* (Lyons et al., 1994; Gold et al., 1999a; Constantini and Isaacson, 2000) and to accelerate the rate of nerve regeneration following a sciatic nerve crush or immediate nerve repair (Gold et al., 1994,1995,1999a,1999b; Wang et al., 1997; Doolabh et al., 1999; Jost et al., 2000; Lee et al., 2000). This effect on nerve regeneration appears to involve binding to a binding protein other than the one (FKBP-12) mediating immunosupression: FK506 binding protein 52 (FKBP-52), a chaperone component of mature steroid receptor complexes (Gold et al., 1999a). For clinical application, however, it is important to determine whether the drug is effective after chronic nerve injuries. Therefore, we examined the effects of FK506 at the same dose (5mg/kg rat weight) as was used in the experiments that examined its effects after nerve crush and immediate nerve repair, on motor axonal regeneration under two conditions of delayed nerve repair (chronic axotomy and chronic denervation) to ask the specific questions whether 1) FK506 will increase the number of motoneurons that regenerate axons after delayed nerve repair, 2) FK506 will accelerate the rate of axonal regeneration in these models of chronic nerve injuries. These results have been presented in abstract form (Sulaiman et al., 2000; Voda et al., 2000).

5.2 MATERIALS AND METHODS

5.2.1 Experimental Models (Prolonged Axotomy and Prolonged Denervation)

Cross-anastomosis *in vivo* methods are used routinely in our laboratory to systematically investigate how chronic axotomy and chronic denervation affect regenerative capacity of motoneurons. Prolonged axotomy is induced by cutting the tibial nerve and preventing regeneration for specific periods of time prior to cross-suturing the proximal TIB nerve stump to the distal stump of a freshly cut common peroneal (CP) nerve to direct regenerating axons from the chronically axotomised motoneurons to the freshly denervated flexor muscles. Prolongation of chronic motoneuron axotomy progressively reduced their capacity to regenerate axons to 33% after a 4-6 month chronic axotomy as compared with when motoneurons were not subjected to chronic axotomy (Fu & Gordon, 1995a).

Chronic denervation is induced by cutting the CP nerve and ligating both proximal and distal stumps to innervated muscles thereby subjecting the distal stump to chronic denervation for specific periods of time prior to cross-suturing the proximal stump of freshly cut TIB nerve into the chronically denervated CP distal stump. Similar to the effects of chronic axotomy on motoneurons, chronic denervation denervation of the distal nerve stumps beyond 1 month dramatically reduced the number of freshly axotomised motoneurons which regenerated their axons (Fu & Gordon, 1995b; Sulaiman and Gordon, 2000).

5.2.2 Surgical Procedures

The tibial and common peroneal branches of rat sciatic nerve were used as models of nerve injury and repair, and for nerve-nerve cross sutures. All animal procedures were conducted in accordance with the Canadian guidelines for animal experimentation, and a local animal welfare committee.

5.2.2.1 Chronic Axotomy

Deep anaesthesia was induced with sodium pentobarbitol anaesthesia (30 mg/kg i.p.) in adult female Sprague-Dawley rats weighing between 200-225g and, using aseptic precautions, their right sciatic nerves were exposed to dissect the tibial (TIB) and common peroneal (CP) branches. The TIB nerve was transected 5 mm distal to the trifurcation of the sciatic nerve (figure 1A) and its proximal stump was ligated and sutured to the innervated biceps femoris muscle to prevent regeneration thereby subjecting TIB motoneurons to chronic axotomy (Fu and Gordon, 1995a). Two months later, rats were reanaesthetized and the chronically axotomised TIB proximal nerve stump was sutured to the distal stump of a freshly cut ipsilateral CP nerve inside in a 5 mm silastic cuff (Dow Corning, 0.64 mm I.D., 1.19 mm O.D.) with a single 8-0 suture (Ethicon, Peterborough, ON) and the first injection of FK506 (experimental group) or saline (control group) was administered. Daily subcutaneous injection of 5mg/kg rat weight of FK506 or saline was continued for 21days.

5.2.2.2 Chronic Denervation

The TIB and CP nerve branches of the sciatic nerve were also used in subjecting Schwann cells of the distal nerve stumps to chronic denervation. Adult female Sprague-

Dawley rats were also used for these surgeries, which were performed under aseptic conditions and deep anaesthesia induced by sodium phenobarbital (30mg/kg i.p.). The right CP nerves of 12 rats were cut and regeneration was prevented by ligating both proximal and distal stumps to adjacent innervated muscles (Figure 1A). This way, regeneration of axons from the proximal stump into the distal nerve stump was prevented so that the target muscle and the distal nerve sheath were both denervated. Denervation of the distal nerve sheath was prolonged for periods of 2 months after which cross-suture of the proximal stump of freshly cut ipsilateral TIB nerve and the distal denervated stump of CP was performed, also using silastic cuff (Figure 1B). This was to facilitate regeneration of the axons of freshly axotomised and regenerating TIB motoneurons through the chronically denervated distal nerve sheaths. Animals were allowed to recover and kept for a period of 21 days during which they received daily subcutaneous injections of 5mg/kg rat weight FK506 (experimental) or saline (control).

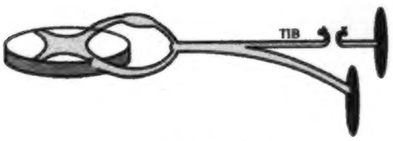
5.2.3 Retrograde Axonal Tracer Application And Histology

Application of retrograde axonal tracers and reinnervated nerve histology have been described previously (Sulaiman and Gordon, 2000). Briefly, 3 weeks following TIB-CP cross-suture and daily injection of FK506 or saline, the CP nerve was cut at a distance of 25 mm from the TIB-CP suture site for application of one of the retrogradely transported fluorescent dyes- fluorogold (FG) and fluororuby (FR) for 1 and 2 hours, respectively. The choice of dye was alternated between animals to control for possible differences in retrograde uptake and transport of the dyes. The skin was then sutured and the animals allowed to recover. Five days later, rats were transcardially perfused with saline and then 4%

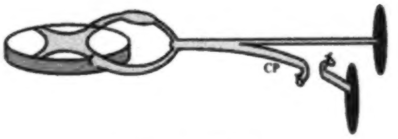
paraformaldehyde in 0.1M PBS. The L2-L6 spinal segment was removed and left in 30% sucrose paraformaldehyde solution overnight. Longitudinal 50um cryostat sections of the spinal cord were cut and examined under the fluorescent microscope. FR-labeled motoneurons were visualized with a Rhodamine filter (580nM), while the FG-labeled motoneurons fluoresced blue under UV light with a 430nM filter. Each longitudinal section was examined and all labeled motoneurons counted (see figure 2).

Nerve histology was concluded as previously described (Wang et al., 1997). Briefly, prior to the application of fluorescent dyes, 3-mm pieces of nerves were excised and fixed by immersion in gluteraldehyde (3% in 0.1M phosphate buffer), stained with osmium tetroxide (3% solution in 0.1M phosphate buffer), dehydrated in ascending alcohols and embedded in araldite. Semithin sections (0.5 um) were stained with toluidine blue; thin sections were stained with uranyl acetate and lead citrate, mounted on film-supported 75 mesh grids, and examined in a JOEL 100X electron microscope. Myelinated and nonmyelinated axons in the entire nerve were counted under an 63X oil immersion lens (final magnification 790X) with the aid of a 100 unit micrometer reticle (10 mm divisions) using a Leitz Laborlux S light microscope.

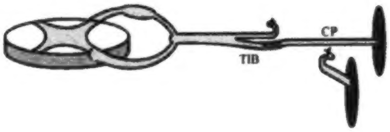
Figure 5.1: A) The TIB nerve was cut and regeneration of the proximal stump was prevented for 2 months (Chronic axotomy) OR the CP nerve was cut and reinnervation of its distal nerve stump was prevented (chronic denervation); B) Two months later, the proximal stump of TIB nerve that was chronically axotomised was sutured to a freshly cut ipsilateral CP + subcut. injection of 5mg/kg/day of FK506 (experimental group) or saline (control group) (old TIB-fresh CP cross-suture) OR the proximal stump of a freshly cut TIB nerve was sutured to the ipsilateral CP nerve that was chronically denervated + subcut. injection of 5mg/kg/day of FK506 (experimental group) or saline (control group) (fresh TIB-old CP cross-suture); C) three weeks following TIB-CP cross-suture and daily injection of FK506 or saline, either one of retrogradely transported fluorescent dyes (FG or FR) was used to backlabel the TIB motoneurons which regenerated axons

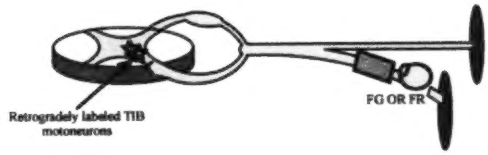


Tibial (TIB) Nerve Cut
(CHRONIC AXOTOMY)



Common Peroneal (CP) Nerve Cut
(CHRONIC DENERVATION)




Retrogradely labeled TIB motoneurons
FG OR FR

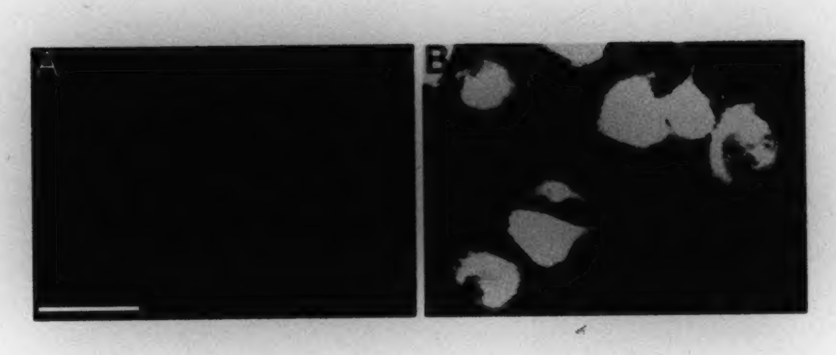
5.2.4 Motoneuron Counting

Using a freezing microtome (Jung CM 3000) each lumbar spinal segment was sectioned into 50μm longitudinal sections as previously described (Sulaiman and Gordon, 2000). Sections were serially mounted on glass slides, allowed to dry and coverslipped. All TIB motoneurons in each spinal cord section that regenerated their axons into the distal CP nerve stumps were retrogradely labelled by the applied fluorescent dyes and could be identified and counted at 20-40X magnification under UV fluorescence. Cells labelled with RR could be easily identified by their red fluorescence and those labeled with FG had blue fluorescence (Figure 2). Only TIB motoneurons in which there was a distinct nucleus and those that could be visualised only under the UV fluorescent filter specific for the dye with which they were labelled were counted; multiple counting of split neurons was corrected for by the method of Abercrombie and Johnson (1946).

5.2.5 Statistical Analysis

A one-way analysis of variance (ANOVA) and the Student's *t*-test were used to compare the mean number of TIB motoneurons that regenerated axons and the respective number of regenerated myelinated and unmyelinated axons in the chronic axotomy and chronic denervation groups. Statistical significance was accepted at the 5% level ($p<0.05$).

Figure 5.2: Photomicrographs of motoneurons backlabelled with fluororuby (A) or fluorogold (B) as viewed under fluorescent microscope. Both fluorescent dyes were able to the somas and dendrites of regenerated TIB motoneurons. Scale bar is 30um.


A
B

5.3 RESULTS

Daily injections of the rats in the experimental groups with FK506 for 3 weeks did not have any apparent generalized secondary effects on their health. Likewise, we encountered no infections or unexpected deaths during the duration of injection, indicating that the immunosuppressive effects of FK506 were tolerated by the animals at this dose and relatively short period of injection.

5.3.1 TIB Motoneuronal Regeneration after 2 Month Chronic TIB Motoneuron Axotomy

Retrograde fluorescent labeling, which labels only neurons that regenerate their axons up to the point of application of the applied fluorescent dyes, was used to identify and count the number of 2 month chronically axotomised TIB motoneurons that regenerated their axons into freshly cut distal CP nerve stumps (with or without daily injections of FK506). The two chosen fluorescent dyes, FG and FR, brightly label both the somas and dendrites of the 2 month chronically axotomised TIB motoneurons that regenerated their axons into the freshly cut CP distal nerve stumps (figure 2).

Two month chronic axotomy of motoneurons has been shown to reduce both the number of TIB motoneurons which regenerated axons into freshly cut distal CP nerve stumps (Boyd et al., 1998) and the number regenerated axons which reinnervate the denervated tibialis anterior muscle by about 66% after TIB-CP cross-suture, compared to the corresponding numbers when immediate TIB-CP cross-suture was performed (Fu and

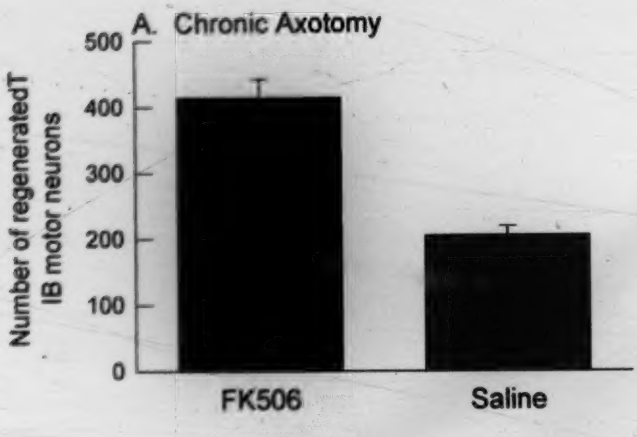
Gordon, 1995a). Therefore, we chose to chronically axotomise TIB motoneurons for 2 months in our experiments so that a positive effect of FK506, if present after delayed nerve repair, could be easily detected. Furthermore, the control group in our experiments, which received daily injection of equal volume of saline, demonstrate a reduced number of TIB motoneurons that regenerated their axons after a 2 month chronic axotomy, [205 ± 27 (mean ± SE)], which is only 24% of the total number of TIB motoneurons in intact TIB nerve. This represents a 76% decrease compared to the 66% previously reported (Fu and Gordon, 1995a). However, the duration of regeneration in these two experiments differ; tibial motor axonal regeneration was estimated after at least 6 weeks in our previous experiments (in which there was 66% reduction), whereas in the present experiments, this was done after only 3 weeks to minimize the duration of injections of FK506. In addition, we noted that it takes more than 6 weeks (up to 12 weeks) for all TIB motoneurons to regenerate their axons after immediate nerve repair (unpublished results), and 8-10 weeks for all femoral motoneurons to regenerate their axons across the surgical site (Al-Majed et al., 2000). Thus, examination of an earlier (3 weeks) time-point enabled us to maximize the ability to observe a discernable effect of the drug on nerve regeneration.

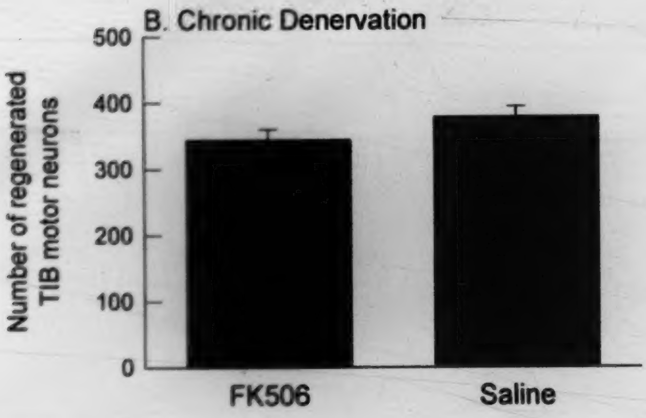
5.3.1.1 Effect of Daily Subcutaneous Injections of 5mg/kg Fk506

Only 205 ± 27 TIB motoneurons regenerated their axons after 2 month chronic axotomy and cross-suture to freshly cut distal CP nerve stump in animals which received daily subcutaneous injections of equal volume of saline (Figure 3A; control group). In contrast, in animals that received daily injections of FK506, 414 ± 62 TIB motoneurons regenerated their axons (Figure 3A). This represents almost a doubling of the number of TIB motoneurons that regenerated axons, compared to the control group, after only 3 weeks regeneration period and treatment with FK506. Furthermore, the data indicate that 50% of all TIB motoneurons regenerated after FK506 treatment which is significantly higher than the 34% that regenerated even after 6 weeks of regeneration time (Boyd et al., 1998). Our findings that about half of the TIB motoneurons regenerated into the distal CP nerve stumps after only 3 weeks is even more impressive when one considers that, assuming a progressive increase in the number of TIB motoneurons that regenerate axons after 2 month axotomy, only 17% of all TIB motoneurons would normally be expected to regenerate after 3 weeks of regeneration. Therefore, FK506 very markedly increased the number of 2 month chronically axotomised TIB motoneurons that regenerated their axons into freshly cut distal CP nerve stumps after a very short duration (3 weeks) of regeneration.

In the reinnervated distal CP nerve stumps, the total numbers of regenerated axons (myelinated and non-myelinated) and the number of the myelinated axons only were determined. Interestingly, the total number of regenerated axons in the FK506-treated animals mirrored the effects on the number of motoneurons in that FK506 also doubled the total number of regenerated axons compared to the saline-treated animals; mean values (± SE) increased from 33 ± 6 um^2 in saline-treated to 76 ± 12/1000 um^2 in FK506-treated animals. Hence, there is direct correlation between the effects of FK506 on increasing the number of TIB motoneurons which regenerated axons and the total number of regenerated axons. The number of myelinated axons was also determined as an indication of the effect of FK506 on

Figure 5.3: A) Numbers (mean ± SE) of 2 month chronically axotomized TIB motoneurons which regenerated axons into freshly cut distal CP nerve stumps were compared when animals received daily subcutaneous injections of FK506 (black bars) or saline (grey bars) (5mg/kg rat weight). Note the significant increase in the number of regenerated TIB motoneurons with FK506 treatment; B) Numbers of freshly axotomized TIB motoneurons which regenerated into 2 month chronically denervated CP distal nerve stumps compared under conditions, as in A. FK506 did not exert any effect on the number of TIB motoneurons that regenerated axons. Statistical analysis by one-way analysis of variance and Student's t test.


A. Chronic Axotomy
Number of regeneratedT IB motor neurons
500
400
300
200
100
0
FK506
Saline

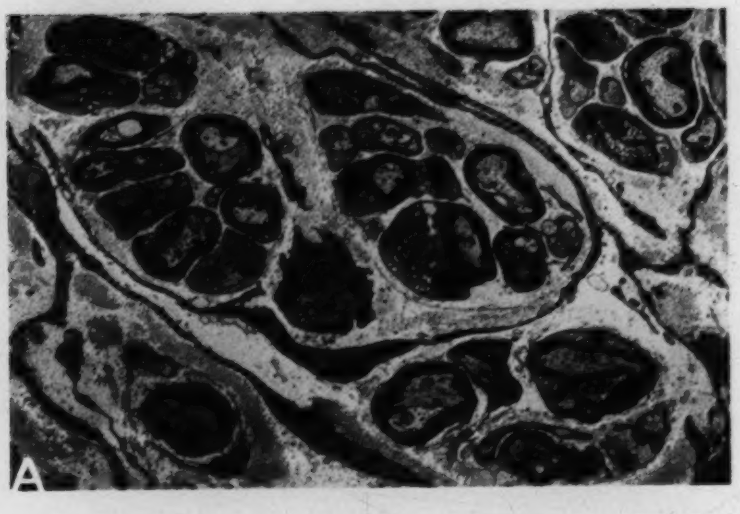

B. Chronic Denervation
Number of regenerated TIB motor neurons
500
400
300
200
100
0
FK506
Saline

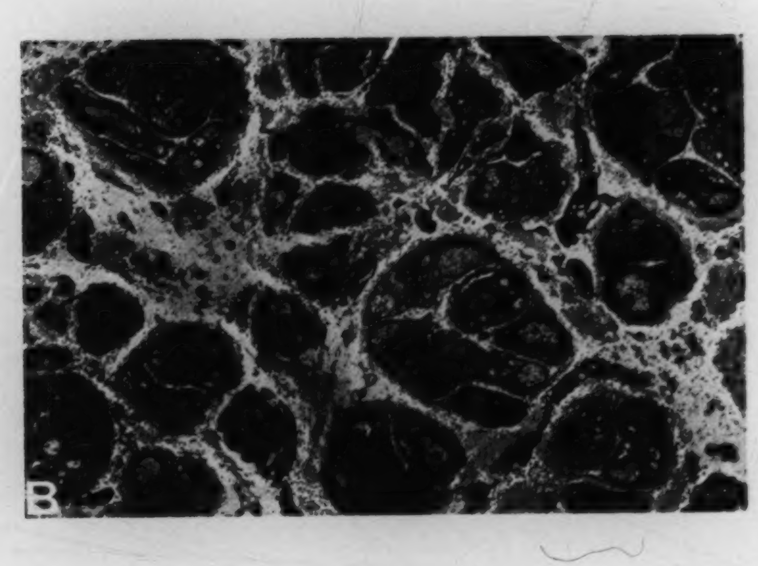
the rate of axonal regeneration; axons whose reinnervation of the distal nerve stump is accelerated should induce the myelinating phenotype of Schwann cells earlier than the later arriving axons and would, consequently, exhibit more extensive myelination. In parallel with its effect of increasing the total number of regenerated axons, FK506 also increased the number of myelinated axons to 93 ± 10 um^2 compared to 57 ± 10 um^2 in the control group (see figures 4 & 6). Taken together, FK506 increased the rate of regeneration (increased number of mature, myelinated axons; Figure 4) and promoted the growth (increased total number of regenerated axons) of regenerating axons in the distal CP nerve stump (Figure 6).

5.3.2 TIB Motoneuronal Regeneration after 2 Month Chronic Schwann Cell Denervation

In a second group of rats, retrograde neuroanatomical tracers and nerve histomorphometry were used to estimate the effects FK506 on the capacity of freshly axotomised TIB motoneurons to regenerate axons into 2 month chronically denervated Schwann cell of the distal CP nerve stump (see Materials and Methods). In contrast to its effects on the increasing regenerative capacity of 2-month chronically axotomised TIB motoneurons, FK506 did not significantly change the number of TIB motoneurons that regenerated axons into the 2 month chronically denervated distal CP nerve stumps; mean (± SE) values were 344 ± 33 in FK506-treated and 378 ± 33 in saline-treated animals (figure 3B). Since there was direct correlation between the numbers of backlabelled TIB motoneurons and the total number of regenerated axons, and no observable difference could be seen in the level of myelination of regenerated axons (figure 4B), we did not expect any

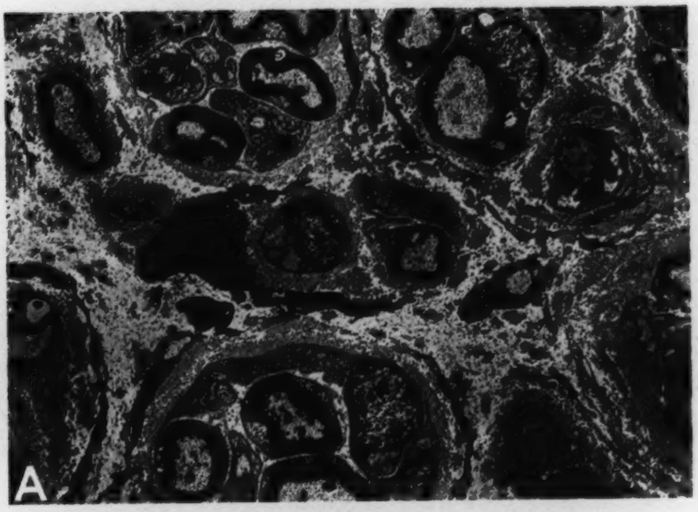
Figure 5.4: Photomicrographs of TIB nerve fibres which reinnervated the distal CP nerve stumps when animals were treated with either FK506 (A) or saline (B) in the chronic axotomy group. Note that TIB axons from animals which receive daily injections of FK506 appear larger and are ensheathed by thicker myelin. This shows that regenerated TIB axons in the FK506-treated animals are in a more advanced state of regeneration than TIB axons from the saline-treated animals. Magnification X 2700.


A


B

difference in the total number of regenerated axons and number of myelinated axons between the control and experimental groups. In contrast, FK506 increases axonal regeneration of freshly axotomised peripheral neurons (Gold et al., 1994, 1995; Wang et al., 1997; Doolabh et al., 1999; Jost et al., 2000; Lee et al., 2000) and chronically axotomised motoneurons (present study). Thus, we suggest that the lack of effect in the chronic denervation injury model is due to its inability to improve the reduced capacity of 2 month chronically denervated Schwann cell to support TIB axonal regeneration.

Figure 5.5: Photomicrographs of TIB nerve fibres which reinnervated the 2 month chronically denervated distal CP nerve stumps with (A) or without (B) FK506 treatment. There is no difference in the thickness of myelin sheaths around the regenerated TIB axons in both groups. Magnification X 2700


A

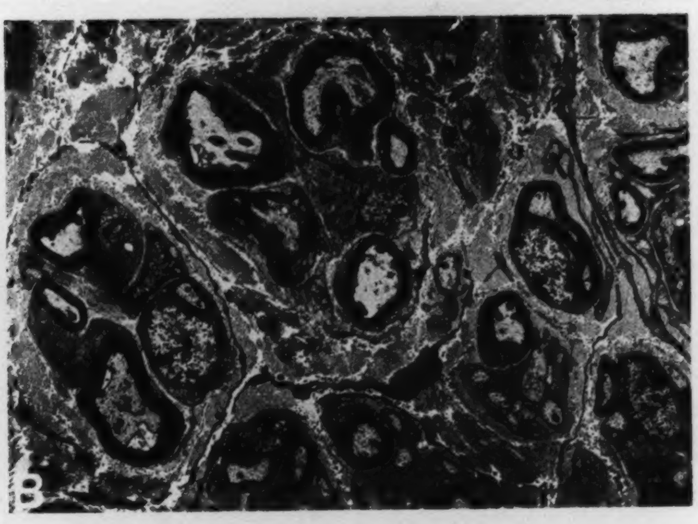

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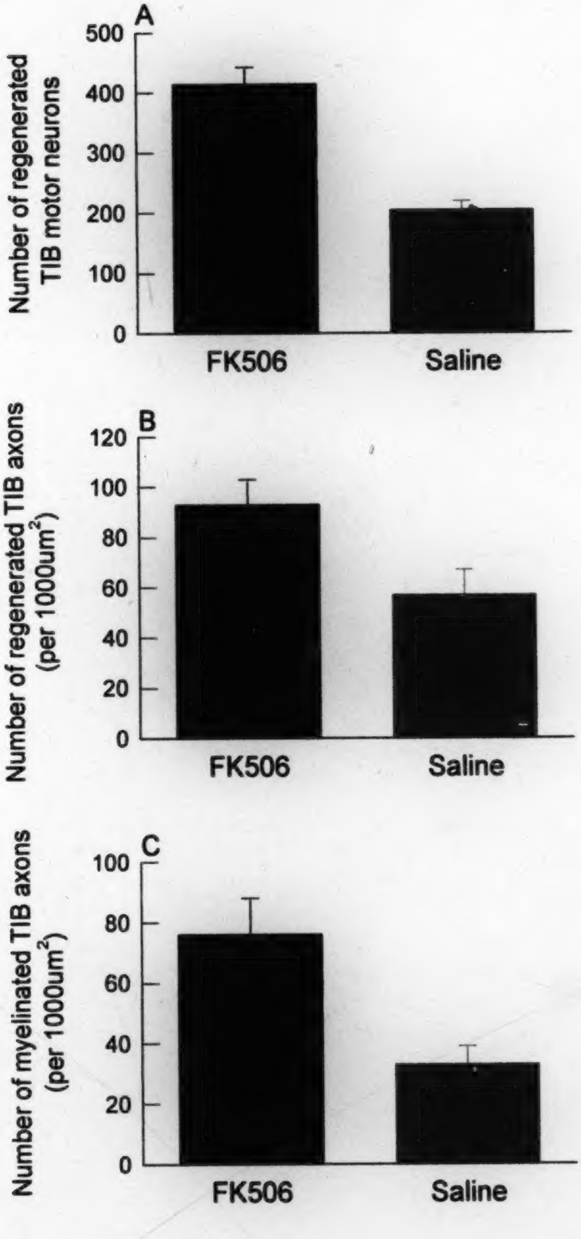
Figure 5.6: Numbers (mean ± SE) of A) 2-month chronically axotomised TIB motoneurons that regenerated axons, B) all regenerated axons and C) myelinated axons in the FK506-treated animals compared to saline-treated animals. FK506 increased the capacity of chronically axotomised motoneurons to regenerate axons (A), increased both the extent (B) and rate (C) of axonal regeneration.

CHRONIC AXOTOMY



5.4 DISCUSSION

This study is the first to demonstrate, using a direct and quantitative neuroanatomical assay, that daily subcutaneous injections of 5mg/kg FK506 after TIB-CP cross-suture i) can reverse the detrimental effect of 2-month chronic axotomy on the capacity of TIB motoneurons to regenerate axons, ii) cannot reverse the effect of 2 month chronic denervation on the capacity of Schwann cells of the distal nerve stump to support axonal regeneration. FK506 increased a) the number of TIB motoneurons that regenerated axons and the total number of regenerated axons in the distal CP nerve stump (thus promoting growth) and, b) the rate of axonal regeneration as measured by the increased number of mature, myelinated axons. In essence, FK506 is effective in improving axonal regeneration after delayed nerve repair, during which motoneurons are chronically axotomised.

Functional recovery after peripheral nerve injury is often disappointing regardless of the capacity of the glial cells of the distal stumps of injured nerves, the Schwann cells, to provide growth-permissive environment. Clinical experience has established that functional recovery is particularly poor for injuries which sever large nerve trunks such as brachial and lumbar plexus injuries (Sunderland, 1978; MacKinnon 1989; Millesi, 1990; Fu and Gordon, 1997). This is because regenerating axons have to travel over long distances to reinnervate denervated targets which they do at very slow rate (1-3mm/day). The slow rate of regeneration may require many months before regenerating axons might be expected to reach denervated targets, during which the motoneurons are chronically axotomised and the growth-supportive environment of the distal nerve stumps is lost (Fu and Gordon, 1997). Hence, faster rates of regeneration are required to eliminate the detrimental effects of chronic axotomy on neurons and to achieve timely reinnervation of the distal nerve stumps, while the Schwann cells are still capable of supporting axonal regeneration.

In the present study, we report that FK506 increases both the rate and extent of axonal regeneration of chronically injured neurons after delayed nerve repair. Although direct estimation (using retrograde labeling) of the number of regenerated neurons after 2-month chronic axotomy in FK506- and saline-treated animals was restricted to motoneurons in our study, we showed that FK506 also increased the total number of regenerated axons in the distal CP nerve stumps. This effect of increasing the number of regenerated axons paralleled the increase in the number of 2-month chronically axotomised motoneurons that regenerated axons with FK506 treatment. Therefore, the effect of FK506 on motoneuronal regeneration could be extended to sensory neurons as well since there is a proportionate increase in the total number of axons (both motor and sensory) which reinnervated the distal CP nerve stumps.

The ubiquitous distribution of the immunophilins in the nervous system (Steiner et al., 1992) suggest that their ligands, including FK506, may play significant roles in the nervous system. Accordingly, it was found that systemic administration of FK506 improved functional recovery following a nerve crush or immediate nerve repair by accelerating the rate of axonal regeneration (Gold et al., 1994,1995; Wang et al., 1997; Doolabh et al., 1999; Jost et al., 2000;Lee et al.,2000).FK506 has also been shown to exert neuroprotective/neuroregenerative effects (for review see Gold, 2000) following ischemic insults (Sharkey and Butcher, 1994; Drake et al., 1996), in spinal cord injuries (Madsen et al., 1998; Bavetta et al., 1999; Wang et al., 1999), and in models of degenerative diseases (Kitamura et al., 1994; Constantini et al., 1998). However, none of these studies specifically investigated whether FK506 could exert its effects of improving functional recovery after delayed nerve repair, which is of clinical significance since immediate nerve repairs are nothing but exceptions in clinical practice. Our study was designed to specifically address this question using an outcome measure which is very direct and quantitative.

The fact that we observed a positive effect of FK506 in promoting motoneuronal regeneration and accelerating axonal regeneration only after the chronic axotomy injury model strongly suggest that the effect of FK506 is mediated via the neuron itself (either at the level of the neuronal cell body and/or proximal axon). This is consistent with studies showing that FK506 promotes neurite outgrowth in PC12 cells, sensory ganglia and hippocampal neurons *in vitro* (Lyons et al., 1994; Gold et al., 1999), and by the upregulation of FKBP-12 mRNA in axotomised motoneurons (Lyons et al., 1992) although a different immunophilin, FKBP-52, appears to mediate the neurotrophic effect of FK506 (Gold et al., 1999). Growth associated genes such as tubulin, actin and GAP-43 are upregulated in the motoneurons after axotomy, however, their expression are lost if motoneurons remain axotomised for a long time (Tetzlaff et al., 1991). The expression of GAP-43, a protein which is essential for growth cone formation and axonal elongation, was increased by FK506 neurons both after spinal cord and peripheral nerve injuries (Gold et al.,1998; Madsen et al., 1998). Therefore, the positive effect of FK506 on motoneurons may be mediated by maintaining (after crush injury or immediate nerve repair) or re-inducing the upregulation of growth-associated genes (after chronic axotomy). Whether FK506 directly induces the upregulation of these genes or indirectly through intermediate mediators remain to be elucidated, although both have been proposed.

In this study, we have provided experimental evidence to clearly demonstrate that FK506 is effective in promoting peripheral nerve regeneration after chronic injuries or delayed nerve repairs. Although more mechanistic studies are warranted, the results of our study suggest that application of FK506 as an adjunct treatment after peripheral nerve injury and repair could be an effective way to improve functional recovery after peripheral nerve injuries. Recently, application of FK506 has been shown to speed functional recovery in two successful hand transplantations (Dubernard et al., 1999; Jones et al., 2000) but more standardized clinical trials are required to further assess the efficacy of FK506 in promoting nerve regeneration after injuries in humans.

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CHAPTER 6

6.0 TRANSFORMING GROWTH FACTOR-β AND FORSKOLIN ATTENUATE THE ADVERSE EFFECTS OF LONG-TERM SCHWANN CELL DENERVATION ON PERIPHERAL NERVE REGENERATION IN VIVO

Adapted from the original publication:

Sulaiman OAR, Gordon T

Glia (In print, August 2001)

6.1 INTRODUCTION

Injured peripheral nerves can regenerate axons although this regenerative capacity does not always translate into good functional recovery (for review see Fu and Gordon, 1997). Clinical experience has shown that even after excellent microsurgical repair, functional recovery is often poor, especially after injury to large nerve trunks which require axonal regeneration over long distance (Sunderland, 1978; MacKinnon, 1989; Millesi, 1990; Terzis & Smith, 1990; Gordon & Fu 1997; Allan, 2000). Regeneration over long distance which occurs at a very slow rate (1-3mm/day) creates a situation in which the motoneurons remain chronically axotomised and the Schwann cells of the distal nerve stump are chronically denervated.

Deterioration of the capacity of Schwann cells to support axonal regeneration after 4 weeks coincides with the decline in the number of infiltrated macrophages and completion of Wallerian degeneration (Liu et al., 1995; Sulaiman & Gordon, 2000). Likewise, the expression of the markers of the growth-promoting, non-myelinating phenotype of Schwann cells such as the erbB receptors, decline after 4 weeks (Li et al., 1997). Thus, it seems that Schwann cells can maintain their growth-supportive phenotype only in the presence of active Wallerian degeneration during which they can interact with infiltrated macrophages (Perry et al., 1987). Infiltrated macrophages play a crucial role in the process of Wallerian degeneration by removing axonal and myelin debris (Beuche and Friede, 1984) and producing cytokines that stimulate production of neurotrophic factors by non-neuronal cells of the distal nerve stumps (Heumann et al., 1987). Transforming growth factor-beta (TGF-β) is among the most widespread and versatile cytokine in the nervous system (reviewed by Bottner et al., 2000; Unsicker & Strelau, 2000). After nerve injury, TGF-β is secreted into the injured nerves by invading macrophages (Assoian et al., 1987) and by Schwann cells themselves (Ridley et al., 1989; Constam et al., 1992). Several *in vitro* evidence have implicated TGF-β in the maintenance of the non-myelinating, growth-promotingSC phenotype (Eccleston et al., 1989; Rogister et al., 1993; Morgan et al., 1994; Chandross et al., 1995; Einheber et al., 1995; Guenard et al., 1995; Stewart et al., 1995a, b; reviewed by Unsicker & Strelau, 2000). Also, TGF-β has been shown to be essential for the neurotrophic effect of several neurotrophic factors, including a very potent neurotrophic factor for motoneurons, glial cell line-derived neurotrophic factor (GDNF; Krieglstein et al., 1998; Schober et al., 1999; reviewed by Unsicker & Krieglstein, 2000). However, the role of TGF-β in axonal regeneration *in vivo* after nerve injury is poorly understood.

We have shown recently that poor functional recovery after nerve injury is primarily due to the progressive deterioration of the capacity of chronically denervated SCs to support axonal regeneration and not the inability of denervated muscles to accept reinnervation as previously believed (Fu and Gordon, 1995a; Sulaiman and Gordon, 2000). In the present studies, we sought to better characterise the specific effects of varying the durations of chronic denervation and regeneration on the number of tibial motoneurons which regenerate their axons over a fixed distance of 25mm using a silastic tubing to stabilise the nerve cross-sutures. The silastic tubes help direct all regenerating axons to the chronically denervated distal nerve. We also investigated the capacity of long-term chronically denervated SCs that have been incubated *in vitro* with TGF-β and forskolin for 48 hours to support axonal regeneration *in vivo*.

This study shows that i) the use of silastic cuffs to stabilise nerve cross-sutures increased the number of TIB motoneurons that regenerated into long-term chronically denervated nerve stumps; ii) after short-term chronic denervation (≤4 weeks), period of between 1.5-3 months is required for all tibial motoneurons to regenerate their axons over a distance of 25 mm, iii) both shorter duration of regeneration and chronic denervation, reduced the number of motoneurons that regenerated their axons over the same distance iv) *in vitro* incubation of long-term chronically denervated SCs with TGF-β and forskolin increased their capacity to support axonal regeneration of tibial motoneuron *in vivo*.

6.2 MATERIALS AND METHODS

Nerve cross-sutures were done inside silastic tubes to direct regeneration of axons of freshly axotomised tibial motoneurons into freshly cut or chronically denervated common peroneal distal nerve stumps.

6.2.1 Surgical Procedures and Nerve Explant Preparation

6.2.1.1 Tibial-Common peroneal nerve cross-suture:

The common peroneal (CP) and tibial (TIB) branches of the right sciatic nerve of adult female Sprague-Dawley rats were used as a model of traumatic nerve injury to study TIB nerve regeneration, after cross-suture, into freshly cut and chronically denervated CP distal nerve stump (Figure1). Under aseptic conditions, and deep anaesthesia induced by sodium phenobarbital (30mg/kg i.p.), the right CP nerves of 60 rats were cut and regeneration prevented by ligating both proximal and distal stumps to adjacent innervated muscles (Fugure. 1A). This way, regeneration of axons from the proximal stump into the distal nerve stump was prevented, leaving the SCs of the distal nerve stumps chronically denervated for periods of 0day, 1 week, 4 weeks, 8 weeks, 12 weeks, 16 weeks, 20 weeks and 24 weeks (n=5) prior to reinnervation.

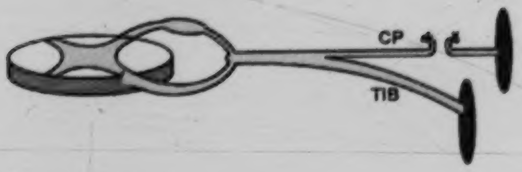
At the end of each denervation period, cross-suture of the proximal stump of freshly cut ipsilateral TIB nerve and the distal denervated stump of CP was performed inside a silastic cuff (Dow Corning, 0.64 mm I.D., 1.19 mm O.D.; Figure 1B) to facilitate regeneration of the axons of freshly axotomised and regenerating TIB motoneurons through the chronically denervated distal nerve sheaths. The use of silastic cuffs was intended to minimize misdirected regeneration, and subsequent loss of regenerating TIB axons. It may also compensates for the anatomical, and hence size discrepancy between freshly cut and chronically denervated nerves. This way, all TIB motoneurons which regenerated axons send their axons in the direction of the distal CP nerve stumps and their continued regeneration is dependent, at least in part, on the capacity of the SCs of the distal CP stumps to support them. In our previous experiments, we looked at regeneration after 12 months to guarantee reinnervation of denervated muscles. In the present experiments, we allowed only 1.5 and 3 months regeneration time, to allow for regeneration of all TIB motoneurons 25mm into the CP distal nerve stumps. Regeneration in 15 rats from 0day, 12 weeks and 20 weeks was allowed for only 1.5 months, to estimate the effect of shorter duration of regeneration on the number of TIB motoneurons that regenerate axons.

6.2.1.2 In vitro reactivation of chronically denervated Schwann cells:

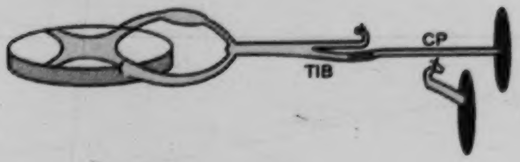
Under aseptic conditions and deep anaesthesia induced by sodium phenobarbital (30mg/kg i.p.), the right sciatic nerves of 6 rats were cut and regeneration was prevented as described above, for a period of 24 weeks (Figure 2A). At the end of 24 weeks, deep anaesthesia was again induced and under aseptic conditions, the chronically denervated right sciatic nerves were dissected out and placed into Liebovitz's L-15 medium (GIBCO) supplemented with 50U/ml penicillin and 0.05mg/ml streptomycin in a 35-mm culture dish. In this medium, the epineurium and connective tissue were stripped off with fine forceps. They were then placed in a fresh L-15/antibiotic medium, minced with scalpel into 2-3mm^2

Figure 6.1: Schematic representation of the tibial (TIB)-common peroneal (CP) nerve cross-suture inside a silastic nerve cuff. A) The CP nerve was cut and the proximal and distal stumps were separated and ligated to prevent axonal reinnervation of the distal CP nerve stump; B) 0-6 months later, the ipsilateral TIB nerve was cut and its proximal stump was sutured, with the aid of silastic cuffs, to the 0-6 month chronically denervated distal CP nerve stumps, in the respective rat groups; C) After either 1.5 (only 0, 3 and 5 months chronic denervation groups) or 3 months (all denervation groups), the number of TIB motoneurons which regenerated their axons into the CP distal nerve stumps was estimated by applying fluorescent dyes (FG or FR) to identify and count the motoneurons.

A. CP Nerve cut



B. TIB-CP Cross-suture



C. Retrograde labelling

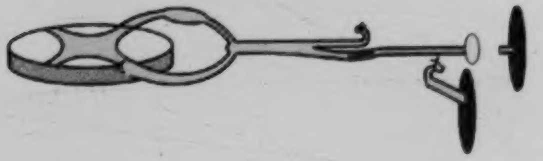
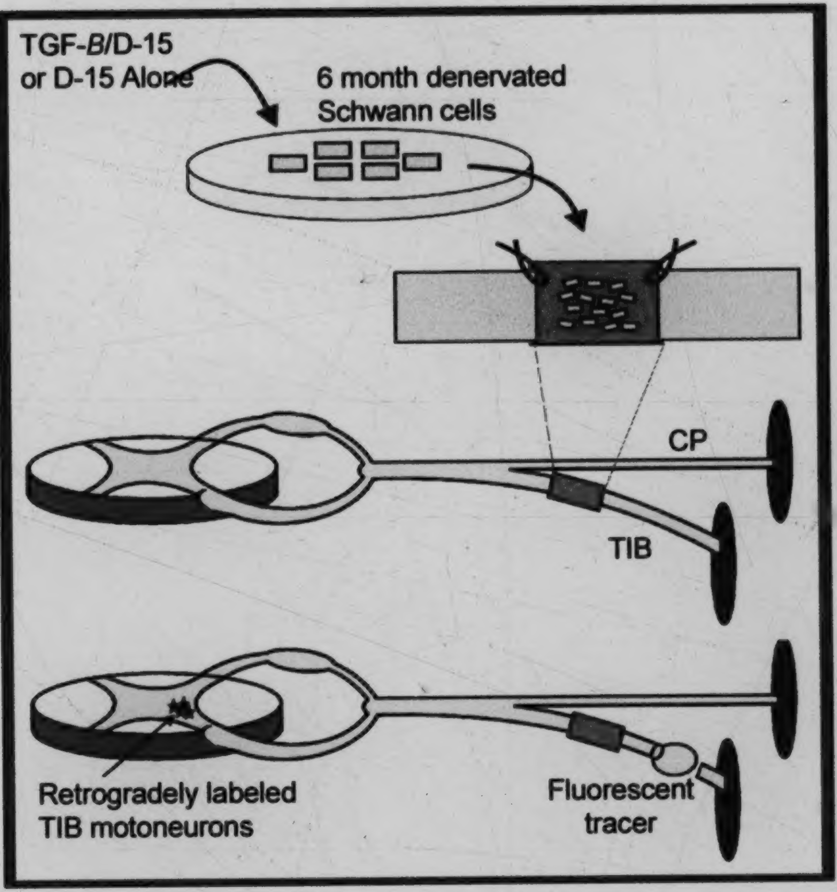


Figure 6.2: Six-month chronically denervated sciatic nerve stumps devoid of epineurium, blood vessels were sliced into 3-4mm^2 *in vitro* and incubated with either TGF-β/D-15 (see method) or D-15 for 48 hours; B) After 48 hours incubation, the nerve explants were placed in silastic cuffs which bridged the gap between the proximal and distal nerve stumps of freshly cut TIB nerves, to encourage axonal regeneration by the freshly axotomised TIB motoneurons through the nerve explants and into the distal TIB nerve stumps; C) 6 months after the surgical procedures described in B, regeneration of TIB axons through the nerve explants and 25mm into the distal TIB nerve stumps was assessed by applying retrogradely transported fluorescent dyes (FG or FR) to the cut ends of the distal TIB nerve stumps. TIB motoneurons which regenerated axons were backlabelled, identified in the spinal cord and counted in both groups.


TGF-*B*/D-15
or D-15 Alone
6 month denervated
Schwann cells
CP
TIB
Retrogradely labeled
TIB motoneurons
Fluorescent
tracer

explants, and transferred into another 35-mm dish in Dulbecco's Modified Eagle's Medium (DMEM, GIBCO) supplemented with 15% fecal calf serum and 0.5uM forskolin (D-15). The culture medium is replaced with another D-15 medium that has (experimental group) or has not (control group) been supplemented with 1ng/ml transforming growth fator-β. We used 1ng/ml of TGF-β and 0.5uM forskolin because both induced SC proliferation at these concentrations *in vitro* (Ridley et al., 1989). The nerve explants were left in the TGF-β/forskolin medium for 48 hours prior to their use in the in vivo experiments.

6.2.1.3 Capacity of in vitro reactivated chronically denervated Schwann cells to support axonal regeneration in vivo:

We exposed nerve explants to either TGF in D-15 (experimental group) or to D-15 alone (control) for 48hours after which 5-6 of either the experimental or control explants were placed in a 1cm long silastic tubing which bridged a gap between proximal and distal nerve stumps of a freshly cut tibial nerve in a rat hindlimb (n=10; Figure 2). This way, all regenerated TIB axons have to traverse the nerve explants before they can reinnervate the distal TIB nerve stumps. The explants were placed in such a way that there was 2-3mm gaps between them and the tibial nerve stumps to minimize the influence of SC migration from the stumps on regeneration. Axonal regeneration by freshly axotomised tibial motoneurons through the nerve explants and 25mm into the distal tibial nerve stumps was allowed for 6 months (longer time of regeneration was allowed to compensate for the gaps between the nerve explants and the tibial nerve stumps, and the distance which motoneurons have to regenerate their axons) after which the number of tibial motoneurons that regenerated their axons was estimated using fluorescent neurotracing technique (see below).

6.2.2 Retrograde Labeling of Motoneurons

6.2.2.1 Regeneration into 0-24 week chronically denervated distal nerve stumps after 1.5 or 3 months regeneration

The number of TIB motoneurons that regenerated their axons through the chronically denervated distal CP nerve stump after 1.5 or 3 months were determined by applying retrogradely transported axonal tracers to backlabel TIB motoneurons (Figure1C). Fluorogold (FG; Fluorochrome Inc. Denver) and Fluororuby (RF; Dextran tetramethylrhodamine, Mol. Probes, D-1817, Eugene, OR) were the 2 dyes chosen since they are effectively endocytosed and retrogradely transported (Richmond et al., 1994; Novikova et al., 1997). The CP nerve of each rat was cut 25mm distal to the site of CP-TIB cross-suture for exposure to either FG or FR for retrograde labelling of TIB motoneurons which regenerate their axons into the chronically denervated CP distal nerve stump. The choice of dye was alternated between animals to control for possible differences in retrograde uptake and transport of the dyes.

Backlabeling with FG was done by exposing the tip of the cut CP nerve to 4% FG in 0.1 M cacodylic acid for 1 h (Schmued and Heimer, 1990) in a vaseline well, after which it was extensively irrigated and reflected to a distant portion of the wound. Backlabeling with FR was done by placing the tip of the cut CP nerve on a 1cm^2 weighing paper (Fisher

Scientific Co., USA) with FR crystals for 2 h, then irrigating the nerve and placing it in the opposite corner of the wound to prevent backlabeling of non-TIB motoneurons by diffusion of tracers. Animals were allowed to recover and kept for 5 days to allow for retrograde transport of the applied fluorescent dyes to the neuronal cell bodies.

6.2.2.2 Regeneration through reactivated and non-reactivated nerve explants into freshly cut distal tibial nerve stump

Six months after transplantation of the nerve explants between the 2 stumps of freshly cut tibial nerve stumps, regeneration through the explants and 25mm further down the distal nerve stumps was assessed. The tibial distal nerve stump was cut 25mm away from the distal end of the silastic tube in which the nerve explants were placed, and which connected the two tibial nerve stumps and, either FG or FR was applied to identify and enumerate the number of tibial motoneurons that regenerated axons. Prior to the application of the fluorescent dyes, 3mm pieces of nerve were excised for histological examination of the morphology of regenerated TIB axons in the distal CP nerve stumps. Labelling with either FG or FR was the same as described above.

6.2.3 Tissue Perfusion and Nerve Histology

On the 5th day after the application of the retrograde dyes, rats were again deeply anaesthetized with sodium phenobarbital (30mg/kg i.p.) and perfused first with 100ml of saline followed by 500 ml of ice cold 4% paraformaldehyde (pH 7.4) through the aorta. After perfusion, the part of the lumbar spinal cord segments which contains all the TIB motoneurons (L4-L6) was removed and post fixed in 30% sucrose in 4% paraformaldehyde solution overnight. Tissues were then frozen in liquid nitrogen and stored at -80°C prior to cryostat sectioning. Following application of retrograde dyes and after transcardiac perfusion of rats with paraformaldehyde, 3 mm pieces of nerve sections were also taken from the middle portion of the reinnervated explants for histological assessment. These nerve sections, and nerve sections taken from the reinnervated distal nerve stumps, were fixed in gluteraldehyde (3% in 0.1M phosphate buffer), stained with OsO_4 (3% solution in 0.1M phosphate buffer), dehydrated in ascending alcohols and embedded in araldite. Sections of 1.5um thickness were cut and stained with methylene blue for photomicrographs.

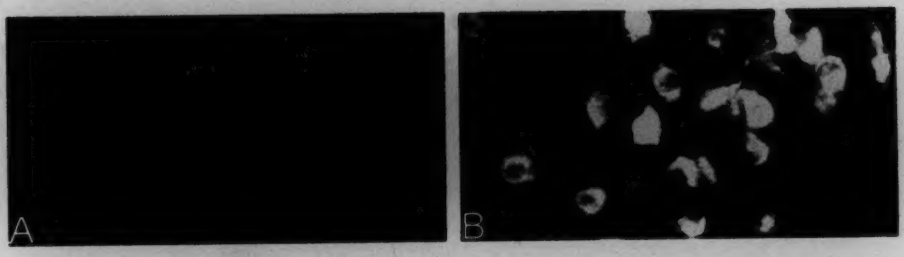
6.2.4 Motoneuron Counting

Each excised lumbar spinal cord segment was mounted horizontally on a cryostat chuck with the dorsal part of the spinal cord facing up for sectioning of 50μm longitudinal sections using a freezing microtome (Jung CM 3000). Sections were serially mounted on glass slides, allowed to dry and coverslipped. Retrogradely labeled motoneurons in each spinal cord section were observed and all the motoneurons counted at 20-40X magnification under UV fluorescence at barrier filters of 430nM for FG and 580nM for FR. Cells labeled with RR could be easily identified by their red fluorescence and those labeled with FG had blue fluorescence (Figure 3). Backlabelled TIB motoneurons which regenerated their axons were identified but only motoneurons in which there was a distinct nucleus and that could be visualized only under the UV fluorescent filter specific for the dye with which they were labelled were counted; multiple counting of split neurons was corrected for by the method of Abercrombie and Johnson (1946).

6.2.5 Statistical Analysis

A one-way analysis of variance (ANOVA) was used to compare the mean number of TIB motoneurons that regenerated axons into the denervated distal nerve stumps after varied length of chronic denervation. Statistical significance was accepted at the 5% level ($P<0.05$). The Student's *t* test was used to compare differences between mean values of number of regenerated TIB motoneurons in the experimental (D-15/TGF-β) and control (D-15) groups with statistical significance acceptable only at the 1% level ($P< 0.01$).

Figure 6.3: Photomicrographs of TIB motoneurons retrogradely labelled with fluororuby (A) and fluorogold (B). Note that both dyes labelled both the somas and dendrites of TIB motoneurons which regenerated axons. Scale bar = 30um


A
B

6.3 RESULTS

6.3.1 Tib Motoneuronal Regeneration into 0-24 Week Chronically Denervated CP Nerve Stumps

In order to establish the time required for all TIB motoneurons to traverse the TIB-CP cross-suture site and 25mm into the CP distal nerve stump, we allowed regeneration for 1.5 and 3 months. The effect of the shorter duration (1.5 months) of regeneration on number of TIB motoneurons which regenerate axons was observed only at denervation time points (0, 12 and 20 weeks) when there are significant changes in the number of TIB motoneuron which regenerated axons (based on our previous study).

6.3.1.1 TIB motoneuron regeneration after 3 months of regeneration time

Axonal regeneration by freshly axotomised tibial motoneurons into 0-24 weeks chronically denervated distal CP stumps was encouraged by TIB-CP cross-suture in a silastic tubing. Three months after TIB-CP cross-suture, either of the two retrogradely transported fluorescent dyes (FG or FR) was used to identify and count the number of TIB motoneurons that regenerated their axons 25mm into the 0-24 chronically denervated CP nerve stumps.

The numbers of TIB motoneurons which regenerated axons into 0-4 weeks chronically denervated CP nerve stumps after 3 months range from 808 ± 7 to 857 ± 24 (black bars in Figure 4A) which were not significantly different from the numbers that regenerated after 12 months regeneration and number of TIB motoneurons in intact nerve, as estimated in our previous experiments (from 994 ± 31 to 1017 ± 23; Sulaiman and Gordon, 2000). This demonstrates that the period of 3 months was sufficient enough time for all TIB motonurons to regenerate their axons 25mm into CP nerve stumps that were chronically denervated for 0-4 weeks, although we did not examine the extent of muscle reinnervation by the regenerated axons. Therefore, it seems like 3 months is adequate amount of time for all TIB motoneurons to regenerate axons but, as shown by Al-Majed et al., (2000), longer period of time may be required for adequate reinnervation of denervated muscles and their recovery from denervation atrophy.

When TIB-CP cross-suture was delayed for more than 4 weeks, there was gradual decrease in the number of TIB motoneurons which regenerated their axons (black bars in Figure 4), consistent with our previous findings (Sulaiman and Gordon, 2000). However, we observed that the use of silastic tubing to consolidate TIB-CP resuture may have contributed to higher numbers of regenerated TIB motoneurons after chronic denervation compared to the numbers in our previous experiments in which TIB-CP cross-sutures were done without the use silastic cuffs, especially after long-term (>1 month) chronic denervation (Figure 4B). Hence, it seems that the use of silastic cuffs partially compensated for the effects of anatomical changes in the chronically denervated CP nerve stumps that otherwise could make their reinnervation by freshly cut TIB nerves somehow inefficient.

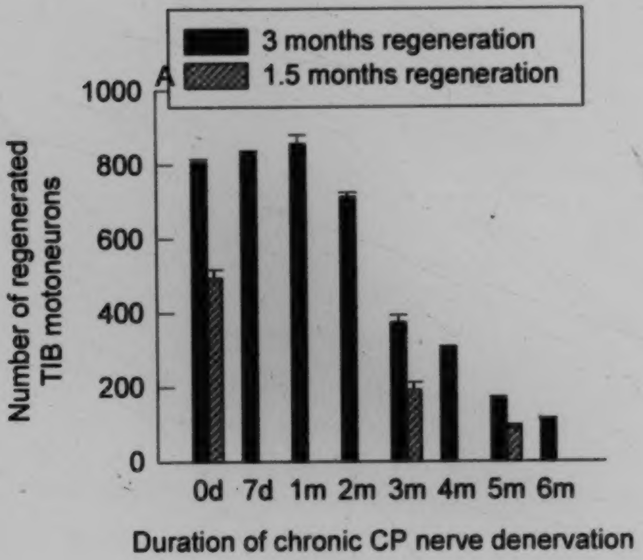
6.3.1.2 TIB motoneuronal regeneration into 0day, 12 weeks and 20 weeks chronically denervated CP nerve stumps after 1.5 months

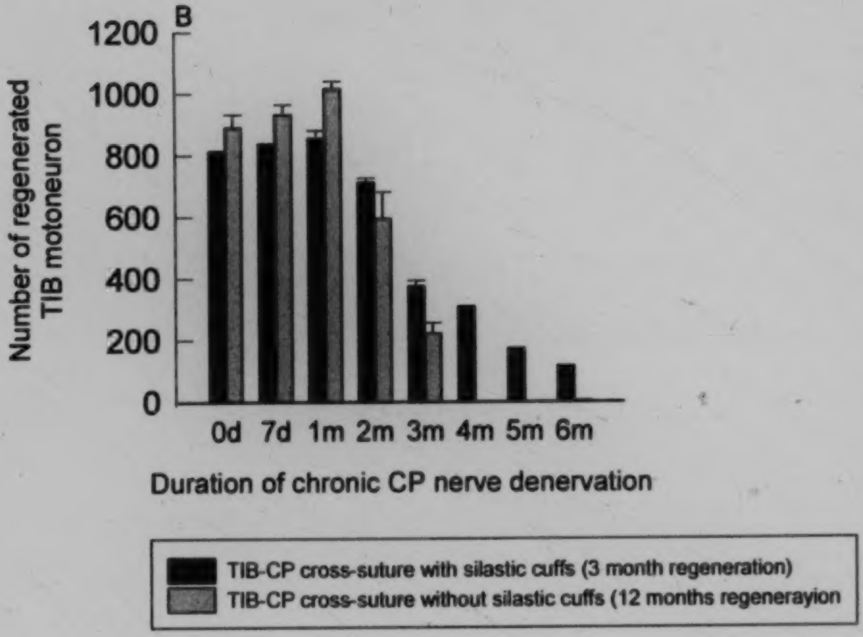
The numbers of TIB motoneurons which regenerated axons into 0, 12 and 20 weeks chronically denervated distal CP stumps were estimated after 1.5 months of regeneration instead of 3 months. Shortening the length of regeneration time reduced significantly the number of TIB motoneurons which regenerated axons both after immediate and delayed nerve repairs (hatched bars in Figure 4A). This effect was more pronounced after longer periods of chronic denervation as the already reduced numbers of regenerated TIB motoneurons were further reduced. This demonstrates that 1.5 months was not sufficient time for all TIB motoneurons to regenerate their axons after immediate. The normally reduced number of regenerated TIB motoneurons after delayed nerve repairs (Sulaiman & Gordon, 2000), was further reduced when regeneration was allowed for only 1.5 months (Figure 4A). Delayed nerve repair beyond 4 weeks also reduced the number of TIB motoneurons that regenerated their axons into the chronically denervated distal CP stumps even after 3 months regeneration time.

6.3.2 Effects of TGF-β/forskolin on the Capacity of Chronically Denervated Schwann Cells to Support Axonal Regeneration

When TIB-CP cross-suture was delayed for more than 4 weeks, the number of TIB motoneurons that regenerated axons was gradually reduced from a mean (± SE) of 808 ± 7 motoneurons after immediate suture to 111 ± 4 after six month delayed nerve repair (Figure 4A, black bars). Since the maximum reduction of number of regenerated TIB motoneurons was observed at six months, we chose this time point to investigate the possibility of improving the capacity of the SCs in 6 month denervated nerve stumps in vitro to support motoneuronal regeneration in vivo.

Figure 6.4: Histograms showing plots of numbers (mean ± SE) of tibial (TIB) motoneurons which regenerated axons into the distal CP nerve stumps as a function of duration of chronic denervation after 1.5 month (stripped bars) and 3 months (filled bars) regeneration time points. The number of TIB motoneurons which regenerated axons was not significantly reduced until following 2 month chronic CP nerve stump denervation and thereafter. The shorter duration of regeneration of 1.5 months significantly reduced the numbers of regenerated TIB motoneurons at the 3 denervation time points (0, 3 and 5 months) points examined. B) The numbers of TIB motoneurons which regenerated axons into the chronically denervated CP nerve stumps when TIB-CP nerve cross-suture was done without the use silastic cuffs (data taken from our previous experiments, filled grey bars) are compared with the numbers in the present experiments in which silastic cuffs were utilised (filled black bars). The use of silastic cuffs for the TIB-CP cross sutures, increased the numbers of TIB motoneurons which regenerated axons into the CP nerve stumps. This effect is more apparent at denervation time points when the numbers of regenerated TIB motoneurons are normally reduced (>2 months), hence, the use of silastic cuffs partially compensate for the deleterious effect of chronic denervation.


3 months regeneration
1.5 months regeneration
A
Number of regenerated TIB motoneurons
1000
800
600
400
200
0
0d
7d
1m
2m
3m
4m
5m
6m
Duration of chronic CP nerve denervation


B
Number of regenerated TIB motoneuron
1200
1000
800
600
400
200
0
0d
7d
1m
2m
3m
4m
5m
6m
Duration of chronic CP nerve denervation
TIB-CP cross-suture with silastic cuffs (3 month regeneration)
TIB-CP cross-suture without silastic cuffs (12 months regenerayion

Nerve explants prepared from sciatic nerves that have been chronically denervated for 6 months were incubated *in vitro* either with D-15 alone or D-15/TGF-β for 48hours prior to testing their capacity to support axonal regeneration *in vivo*. Freshly axotomised TIB motoneuronal regeneration through the nerve explants and 25mm into the distal tibial nerve was assessed after 6 months of regeneration time. When the nerve explants were incubated with only D-15, 258 ± 13 (mean ± SE) TIB motoneurons were backlabelled. This number was significantly higher than when TIB motoneurons regenerated into CP nerve stumps without in vitro treatment (111 ± 4; see Figure 5). However, even more TIB motoneurons regenerated axons (442 ± 23 motoneurons) when the 6 month chronically denervated nerve explants were pre-treated with D-15/TGF-β for 48hours. Therefore, pre-treatment of chronically denervated SCs in nerve explants greatly enhanced their capacity to support regeneration of freshly axotomised TIB motoneurons. In fact, the number of TIB motoneurons which regenerated axons was 4 times the number which normally regenerate into 6 month chronically denervated nerve stumps without pre-treatment (Figure 5).

Examination of the profiles of regenerated TIB axons in the nerve explants and the distal nerve stumps, further illustrate the significant positive effect that prior incubation of chronically denervated nerve stumps with DMEM/TGF-β/forskolin, had on their capacity to interact with regenerating axons. Concurrent with increased number of TIB motoneurons which regenerated axons through the nerve explants that were treated with DMEM/TGF-β/forskolin, observation of the population of nerve fibers in the middle portions of the these nerve explants showed significantly more nerve fibers, which were contained in a nerve fascicle that looked anatomically intact compared to the degenerative appearance of the nerve fibers in the untreated nerve explants (Figure 6A1 and B1). The nerve fibers in the treated nerve explants were significantly larger in size and were better myelinated (Figure 6A2, B2). However, chronically denervated SCs of the untreated nerve explants maintained their capacity to remyelinate regenerated axons, consistent with previous findings (Wood et al., 1998; Sulaiman & Gordon, 2000). These profiles of regenerated nerve fibers in the explants, were maintained distal to the suture sites, as higher population of nerve fibers reinnervated the distal nerve stumps in the experimental (DMEM/TGF-β/forskolin-treated group) compared to the control (D-15-treated) group (Figure 7 A1, A2, and B1, B2). These nerve fibers were also larger in size and were better myelinated (Figure 7A3, B3).

Figure 6.5: Numbers of TIB motoneurons which regenerated their axons through either TGF-β/D-15-treated or D-15-treated nerve explants and 25mm into the distal TIB nerve stumps was enumerated and compared to the number of TIB motoneurons which regenerate axons into 6 month chronically denervated CP nerve stumps (represented as 'none'). Incubation of 6 month chronically denervated sciatic nerve stumps with just D-15 alone increased their capacity to support TIB motoneuron regeneration, but this capacity was most augmented when the nerve explants were incubated with TGF-β and D-15.

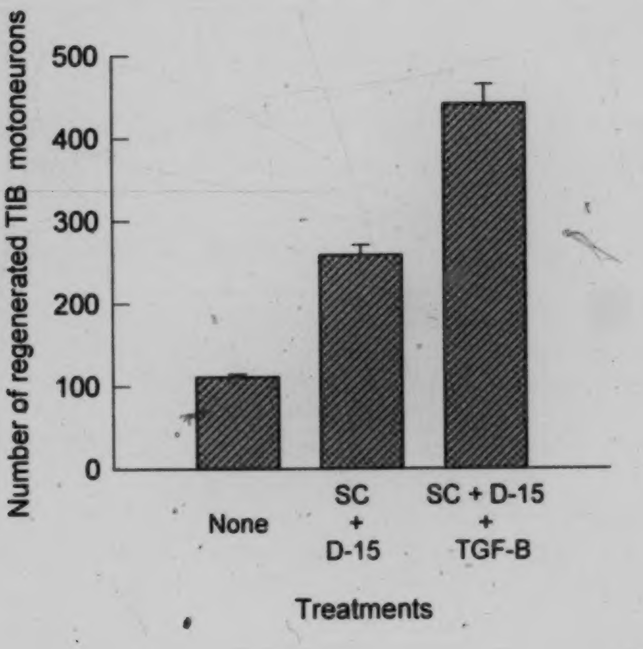
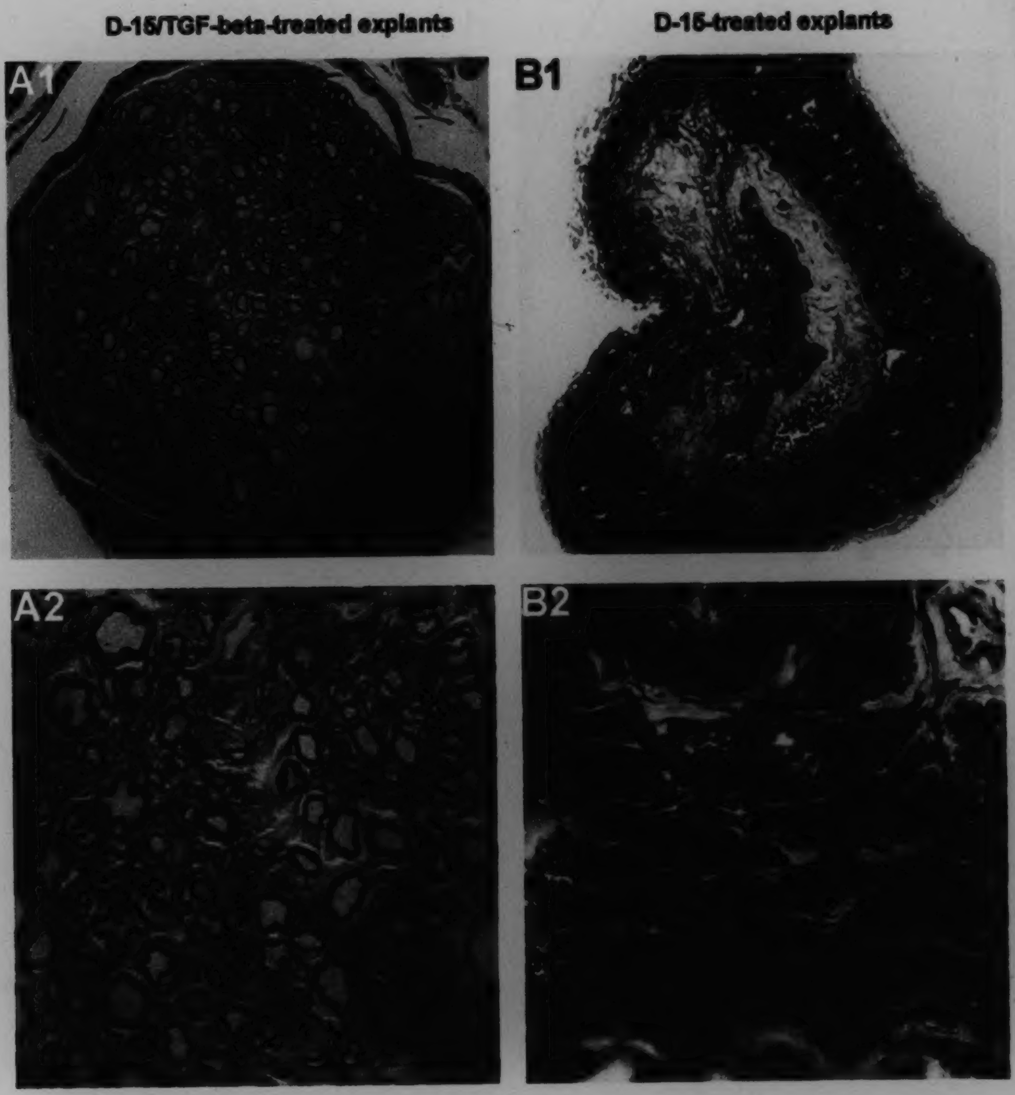
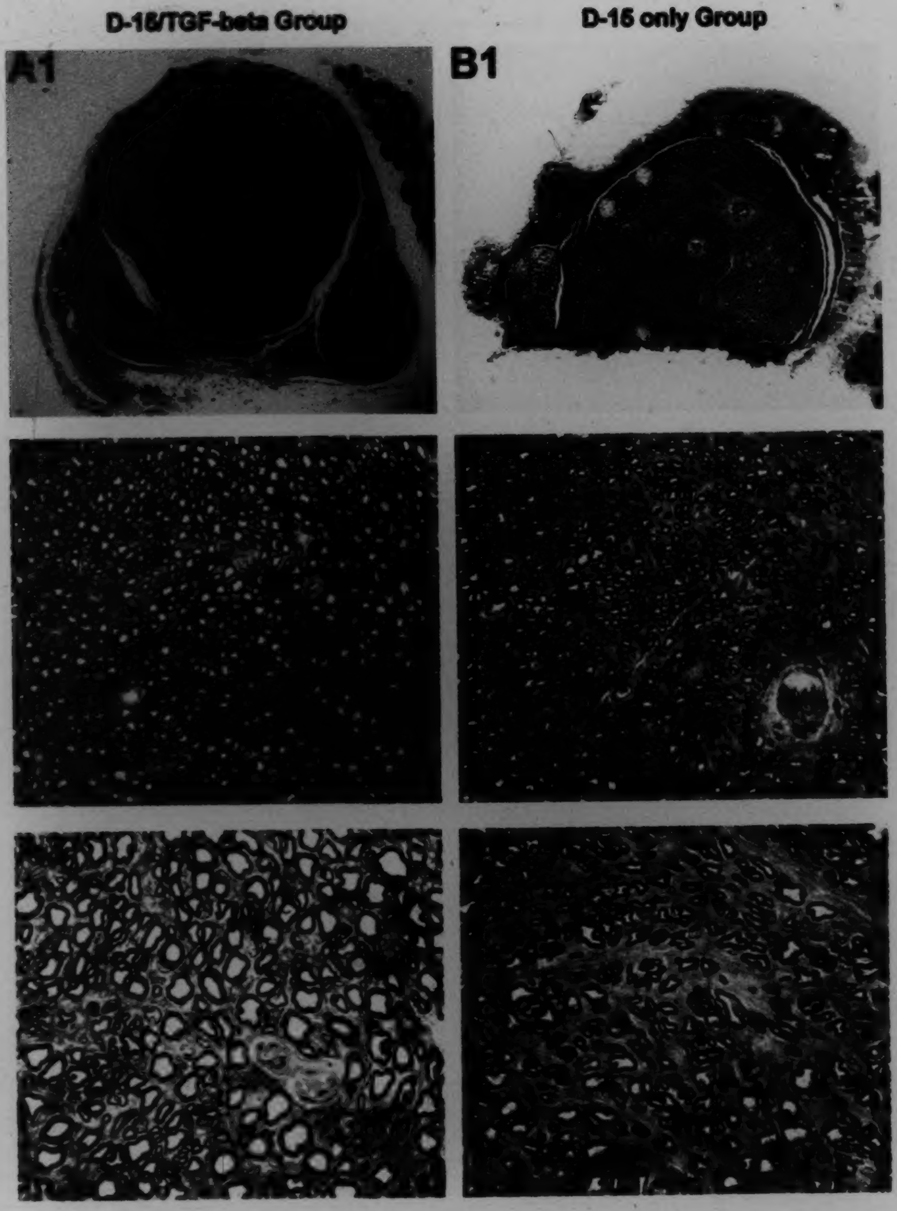

Number of regenerated TIB motoneurons
500
400
300
200
100
0
None
SC + D-15
SC + D-15 + TGF-B
Treatments

Figure 6.6: Light micrographs of reinnervated nerve explants which were (A1, A2) and were not (B1, B2) incubated with TGF-β. Note the normal appearance and myelination of the axons which reinnervated nerve explants that were treated with TGF-β compared with the degenerative appearance of the axons which reinnervated the nerve explants that were treated with D-15 alone. Most regenerated axons in the D-15-treated nerve explants are very small and located on the edges of the nerve explants with a large cavity in the centre of the group of explants (B1), although the regenerated axons were myelinated (B2). Also, greater number of regenerated axons (A1) and thicker myelin (A2) were seen in the TGF-β-treated groups. Magnifications are 40X (A1, B1) and 100X (A2, B2).


D-15/TGF-beta-treated explants

D-15-treated explants

Figure 6.7: Light micrographs of TIB axons which reinnervated the distal TIB nerve stumps having regenerated through either TGF-β-treated (A1-A3) or untreated (B1-B3) nerve explants. As seen in A1 compared to B1, more nerve fibres regenerated into the distal TIB nerve stumps in the TGF-β-treated group. Majority of nerve fibres which regenerated through the TGF-β-treated nerve explants to reinnervate the distal TIB nerve stumps were bigger and well myelinated (compare A2, A3 with B2, B3). Magnifications are 10X (A1, B1), 40X (A2, B2) and 100X (A3, B3).



D-15/TGF-beta Group
D-15 only Group
A1
B1

6.4 DISCUSSION

In this paper, in order to better characterise the effects of chronic distal nerve stump denervation on the capacity of motoneurons to regenerate axons, we performed TIB-CP nerve cross-anastomoses inside silastic cuffs which served to direct regenerated TIB axons into denervated CP nerve stumps and, estimated the number of TIB motoneurons which regenerated axons after 1.5 and 3 months. Also, we investigated the possibility to increase the capacity of SCs that have been subjected to very severe chronic denervation to support regeneration of TIB motoneurons *in vivo* by exposing them to TGF-β and forskolin for 48 hours *in vitro*.

6.4.1 Axonal Regeneration after Immediate and Delayed Nerve Repair

Previously we looked at regeneration of TIB motoneurons into short- and long-term denervated CP nerve stumps after 12 months to allow for regenerated TIB axons to reinnervate denervated muscles and for reinnervated muscles to recover from denervation atrophy (Sulaiman & Gordon, 2000). In these experiments, we found that the numbers of TIB motoneurons which regenerated axons into the distal CP stumps were gradually reduced as the duration of chronic denervation was prolonged. However, the lower numbers of TIB motoneurons which regenerated their axons into the long-term chronically denervated CP nerve stumps could have been due to, at least in part, the degeneration and/or withdrawal of regenerated TIB axons in the nerve stumps by the end of the 12 month regeneration time, and degeneration of regenerated axons as a result of the advanced age of the rats used in the experiments (reviewed by Melcangi et al., 2000). Therefore, in the present experiments, younger rats that were age matched were used. Thus, all animals were the same age at the beginning and at the end of the experiments, and regeneration was allowed for only 1.5 and 3 months to minimise the influence of aging of the rats. Also, regeneration of TIB axons into distal CP nerve stumps was allowed under conditions of optimal guidance of regenerating axons with the use of silastic tubing.

Under these conditions, the effects of chronic denervation on the capacity of motoneurons to regenerate axons were similar to our previous findings (denervation period beyond 4 weeks reduced the number of TIB motoneurons which regenerated axons into the distal CP stumps). However, the use of silastic tubes and probably earlier time of outcome measure, improved the reinnervation of the distal CP nerve stumps after TIB-CP nerve sutures such that the effects of chronic denervation of the number of TIB motoneurons were not as pronounced as when we did not use silastic tubes (Figure 4B; Sulaiman and Gordon, 2000). This positive effect of using silastic tube may be as a result of, 1) improving the alignment of TIB and CP nerves during cross-suture, thereby directing all regenerating TIB axons toward CP distal nerve stumps; 2) reducing aberrant regeneration of TIB axons into surrounding tissues, where these axons could have been lost, and would not have been backlabelled by the applied fluorescent dyes since they did not regenerate into the distal CP nerve stumps; 3) minimizing the effects of anatomical and/or size mismatch between the freshly cut TIB proximal nerve stumps and the shrunken chronically denervated CP distal nerve stumps.

We found that, in order for all TIB motoneurons to regenerate their axons 25mm into CP nerve stumps, longer than 1.5 months regeneration time was required as less number of motoneurons regenerated when regeneration was allowed for 1.5 months. All TIB motoneurons have regenerated their axons after 3 months of regeneration time. Hence, these findings further consolidate our previous findings that it is the deterioration of the growth-supportive SC environment in the distal nerve stumps after delayed nerve repair that accounts, at least in part, for poor functional recovery after peripheral nerve injuries.

6.4.2 TGF-β Improved the Capacity of 6 month-Chronically Denervated Schwann Cells to Support Axonal Regeneration

The initial response of SCs to nerve injury which includes phenotypic change and proliferation, are associated with expression of molecules that promote nerve growth, such as NCAM, L1, GAP-43 and GFAP (Martini & Schachner, 1988; Jessen et al., 1990; Scherer & Salzer, 1996). However, SCs which have been subjected to long-term chronic denervation lose their capacities both to respond to regenerating axons by proliferation (and are therefore gradually reduced in number) and to maintain the endoneurial pathways (Giannini & Dyck, 1990; 1998; Terenghi et al., 1998; Wood et al., 1998; Stoll & Muller, 1999) although they maintained their capacity to remyelinate regenerated axons *in vitro* and *in vivo* (Wood et al., 1998; Sulaiman & Gordon, 2000). Hence, it seems that loss of SC capacity to proliferate and maintain their number is one of the major detrimental factors which account for their inability to support axonal regeneration, especially since they lose the expression of erb B receptors after chronic denervation (Li et al., 1997). These receptors mediate the effects of axonal-derived neuregulin in inducing SC proliferation and motility (Meintanis et al., 2000).

The capacity of TIB motoneurons to regenerate axons was most severely compromised when SCs of the distal CP nerve stumps were chronically denervated for 6 months. Therefore, we chose this time point to investigate whether TGF-β could reverse the deleterious effects of chronic denervation on the capacity of SCs to support axonal regeneration. Six month-chronically denervated distal nerve stump explants were incubated for 48hours with 1ng TGF-β and 0.5uM forskolin which has been shown to augment the effects of TGF-β by increasing intracellular levels of cAMP. A combination of 1ng TGF-β and 0.5uM forskolin was chosen since it has been shown to be most potent in increasing SC proliferation *in vitro* (Ridley et al., 1989). Also, TGF-β has been shown to exert biological effects on SC within 48hours *in vitro* (Rogister et al., 1993; Chandross et al., 1995). Interesting, incubation of 6 month chronically denervated SCs for 48hours *in vitro* with the chosen concentrations of TGF-β and forskolin dramatically increased their capacity to support regeneration of freshly axotomised TIB motoneurons *in vivo*, although incubation with only D-15 was also able to improve the capacity of these SCs to support axonal regeneration compared to if they are not incubated (Figure 5). This positive effect of D-15 on axonal regeneration is not surprising since serum itself contains a mixture of hormones and growth factors (Stewart et al., 1991), and pre-exposure of SCs to serum has been shown to augment their responsiveness to growth factors such as those that are released after nerve injuries (Dong et al., 1997). Likewise, forskolin by itself regulates SC phenotype: in the presence of serum and when SC are in the proliferative state, it suppresses their expression of the myelinating phenotype, and induces the myelinating phenotype in quiescent SCs cultured in serum-free medium (Morgan et al., 1994).

Possible Effects of TGF-β on Chronically Denervated Schwann Cells Based on *in Vitro* Observations

The TGF-βs are a family of polypeptides, believed to be involved in development, wound healing and tumorigenesis in vivo (for review, see Roberts et al., 1988; Unsicker & Strelau, 2000). Most cell types express TGF-β receptors (Wakefield et al., 1987), but the action of TGF-β on cell proliferation in culture varies from inhibition to stimulation, depending on cell type, growth conditions and the growth factors present (Moses et al., 1985; Stewart et al., 1991; Dong et al., 1997). The effects of forskolin and TGF-β on SCs are very complex. They both downregulate the expression of myelinating phenotype by SCs in the presence of serum in vitro (Stewart et al., 1995a, b), although TGF-β has also been shown to have the same effect under conditions in which SCs were co-cultured with DRG neurons in serum-free medium (Guenard 1995a, b). Both forskolin and TGF-β promote the non-myelinating growth-supportive phenotype of SCs, although the effect of forskolin is strongly dependent on whether SCs are in a quiescent or proliferative state (Morgan et al., 1991).

There is relatively little information about the intrinsic physiologic role of TGF-β in vivo, and the exact mechanism of how TGF-β/forskolin could have improved the capacity of chronically denervated SCs to support axonal regeneration was not addressed in these studies. Preliminary results show that they increase the immunoreactivity for erb B3 on long-term chronically denervated SCs in vitro (data not included). However, if indeed the major detrimental effect of long-term chronic denervation was progressive reduction in SC numbers and their atrophy, then mitogenic and possibly other effects of TGF-β might be mediating its positive effect on long-term denervated SCs. It is well known that many SC responses are stimulated by cytokines and mitogenic factors (Wohlleben et al., 1999). Following nerve injury, TGF-β is secreted into the injured nerves by injured dorsal root ganglion (DRG) neurons (Rogister et al., 1993), invading macrophages (Assoian et al., 1987) and by SCs themselves (Ridley et al., 1989). TGF-β has been shown to enhance proliferation of cultured SCs, an effect which is augmented by forskolin (Eccleston et al., 1989; Ridley et al., 1989; Rogister et al., 1993), although Mews and Meyer (1993) did not report a proliferative effect of TGF-β on SCs. Regardless of these opposing reports, incubation of chronically denervated SCs to TGF-β *in vitro* may increase their eventual responsiveness to physiologic proliferative signals *in vivo*, such as axonal plasma membranes. In addition, forskolin in the TGF-β/forskolin medium to which chronically denervated SCs were exposed, has been shown to be able to mimic the mitogenic effects of nerve injury on SCs by binding reversibly to adenylate cyclase and activating protein kinase A to increase intracellular cAMP (Seamon and Daly, 1981), an effect which is more pronounced by the addition of growth factors either as components of the serum or added separately (Stewart et al., 1991).

Central to the repertoire of changes in SCs following nerve injury is change in phenotype from that of myelinating to non-myelinating which involves changes in gene expressions. Myelin-associated proteins such as P0 and myelin-associated glycoprotein (MAG) are downregulated in association with the upregulation of regeneration-associated genes such as the neurotrophin receptor p75NTR, neuregulin and their receptors erb B2, erbBB3 and erbB4, glial fibrillar acidic protein, L1, neural-cell adhesion molecule (NCAM), and growth-associated protein (GAP-43) (Chao et al., 1986; Chen et al., 1994; Carraway and Burden, 1995; Scherer and Salzer, 1996; Carroll et al., 1997; You et al., 1997; Raabe et al., 1998; Rahmatullah et al., 1998;). These changes in gene expression are transient and if reinnervation of the distal nerve stumps is delayed, SCs are unable to maintain the growth-supportive phenotype and lose their responsiveness to regenerating axons (Pellegrino and Spencer, 1985; You et al., 1997). Transforming growth factor-β upregulates the expression of NCAM and L1 in vitro (Stewart et al., 1995b), but downregulates the expression GAP-43 (Stewart et al., 1995a), another marker of the non-myelinating phenoype. The significance of this differential effects is not well understood, although the adhesion molecules, L1 and NCAM, are essential for the axon-SC interactions (Martini & Schachner, 1988; Scherer & Salzer, 1996). Also, TGF-β promoted the non-myelinating growth-supportive phenotype of SCs while blocking their expression of the myelinating phenotype (Morgan et al., 1994; Guenard et al., 1995a, b; Stewart et al., 1995a, b). Hence, incubation of chronically denervated SCs may 'refresh' their non-myelinating phenotype and increase their responsiveness to regenerating axons when they were placed *in vivo*.

SCs are highly specialised for the synthesis of extracellular matrix, and secrete collagen type IV, laminin, and heparan proteoglycans that form the basal lamina which is essential for axonal regeneration (Timpl and Martin, 1981; reviewed by Chernousov & Carey, 2000). However, after chronic denervation SCs lose the capacity to maintain their basement membrane as they disintegrate (Giannini and Dyck, 1990). TGF-β enhances the formation of extracellular matrix, upregulates the expression of collagen type IV mRNA in SCs and prevents disintegration of the basal lamina (Ignotz and Massague, 1986; Roberts et al., 1986; Rogister et al., 1993). These effects of TGF-β may have reversed the adverse effects of chronic denervation on SCs as demonstrated indirectly by the results of our experiments in which exposure of 6-month chronically denervated SCs to TGF-β for 48hours increased their capacity to support regeneration of freshly axotomised TIB motoneurons and maintained the integrity of the endoneurium (Figures 5, 6, & 7).

GDNF is a very potent neurotrophic factor for motoneurons (Henderson et al., 1994) and it is upregulated in the SCs of the distal stumps of injured nerve (Naveilhan et al., 1997). The upregulation of GDNF is associated with a change to the non-myelinating phenotype by SCs and presumably, occurs concurrently as the upregulation of TGF-β in the distal nerves stumps. Whether the upregulations of both factors are directly related is not known, but both in vitro and in vivo evidence have demonstrated that GDNF failed to exert its neurotrophic effects in the absence of TGF-β (Krieglstein et al., 1998; Schober et al., 1999). Immunoneutralisation of endogenous TGF-β in vitro abolishes the neurotrophic effect of GDNF on neuronal culture (Krieglstein et al., 1998), and co-administration of neutralising antibodies to TGF-β, prevented the survival effect that administration of GDNF normally exerts on sympathetic preganglionic neurons after adrenomedullectomy (Schober et al., 1999). The exact effect of incubating chronically denervated nerve explants with TGF-β on their expression of neurotrophic factors such as GDNF was not examined in our studies. However, since there was a significant increase in the number of motoneurons that regenerated axons through the TGF-β-incubated nerve explants, it is possible that TGF-β increased the expression of the very potent motoneuron neurotrophic factor-GDNF, and possibly other neurotrophic factors, in these explants. The direct mechanisms of the positive effects of TGF-β on the capacity of chronically denervated SCs remain unknown.

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CHAPTER 7

7.0 SUMMARY, GENERAL DISCUSSION, CONCLUSIONS AND FUTURE DIRECTIONS

7.1 GENERAL DISCUSSION AND SUMMARY

This section summarises the main findings of this thesis, and discusses the findings in the context of the factors responsible for poor functional recovery after peripheral nerve injuries.

7.1.1 Poor Functional Recovery Despite Capacity of the Peripheral Nervous System to Regenerate Axons

The experiments in this thesis sought to answer the important question of why functional recovery is poor after peripheral nerve injuries despite the intrinsic capacity of injured peripheral neurons to regenerate axons, and the provision of growth-supportive environment by the Schwann cells (SCs). Specifically, we used an experimental model of nerve injury and repair in adult rats to answer the following questions: 1) how does delayed nerve repair, and consequently chronic denervation of the SCs of the distal nerve stumps affects, a) the capacity of injured motoneurons to regenerate axons into the chronically denervated nerve stumps, and b) the responsiveness of the chronically denervated SCs in the distal nerve stumps to molecular signals from regenerating axons, to express neurotrophic factors and their receptors (e.g. GDNF and its receptors), and to re-myelinate regenerated axons; 2) whether chronic denervation of SCs can be prevented by a) simultaneous neurotisation of the SCs in a long sensory autograph with a sensory nerve while a motor nerve regenerate into the other end of the graft; 3) whether motor axonal regeneration after delayed nerve repair can be improved by, in vivo application of the immunophilin FK506, which accelerates the rate of axonal regeneration after immediate nerve repair, and 4) whether in vitro incubation of chronically denervated SCs with TGF-β, which modulates their phenotype and growth, would improve their support of motor axonal regeneration in vivo. The results of the experiments which were designed to answer the aforementioned questions are summarised and briefly discussed below:

7.1.1.1 Chronic Schwann cell Denervation

Clinical experience has shown that primary nerve repair is superior to secondary nerve repair with or without grafting (Kline et al., 1974, 1983, 1986, 1998; Kline, 2000; Millesi, 1984, 1987, 2000). However, even after impeccable primary nerve repair, functional recovery is often poor, especially after repair of nerve trunks which require injured neurons to regenerate axons over long distance such as the brachial or lumbar plexuses (Fu & Gordon, 1997; Allan, 2000). At the slow rate of regeneration of 1-3mm per, reinnervation of the distal nerve stumps and end organs is delayed. Hence, the SCs of the distal nerve stumps and the end organs become chronically denervated. It is generally believed that, it is the profound atrophy of the end organs, especially muscles, that account for the poor functional recovery after nerve injuries since the atrophied muscles fail to accept reinnervation by regenerated axons. However, results from the first set of experiments in this thesis dispute that argument and showed conclusively that, 1) the effects of chronic denervation is more profound on the SCs of the distal nerve stumps, and it is the inability of the chronically denervated SCs to support axonal regeneration by injured motoneurons that accounts for poor functional recovery; 2) atrophy of denervated muscles is a slowly progressive process, such that chronically denervated muscles remain viable even after very prolonged chronic denervation, and can accept reinnervation by axons which regenerate through the distal nerve stumps (Fu & Gordon, 1995a; Sulaiman & Gordon, 2000). Furthermore, we found that SCs that have been subjected to very prolonged chronic denervation, 1) progressively lose their expression of a very potent neurotrophic factor for motoneurons, GDNF, and 2) fail to upregulate the expression of GDNF when reinnervated by regenerating axons, compared to their counterparts that have been subjected to shorter duration of chronic denervation, which upregulates the expression of GDNF when reinnervated by regenerating axons (Hoke et al., 2001). This may account, at least in part, for their inability to support motor axonal regeneration into the distal nerve stumps.

7.1.1.1.1 Wallerian Degeneration Is Essential for Capacity of the Schwann Cells to Support Axonal Regeneration

Chronic denervation of SCs for periods of up to 4 weeks had no adverse effect on their capacity to support motor axonal regeneration (Fu & Gordon, 1995b; Sulaiman & Gordon, 2000). However, chronic denervation of SCs beyond 4 weeks progressively reduced their capacity to support motor axonal regeneration. Interestingly, the period of 4 weeks during which SCs support motor axonal regeneration coincides with the time when, the process of Wallerian degeneration is most active (Liu et al., 1995), and SCs can actively interact with macrophages which infiltrate the injured nerve stump. Likewise, the expressions of growth factors and their receptors by SCs have gradually declined by the end of Wallerian degeneration (Taniuchi et al., 1986; Heumann et al., 1987; Acheson et al., 1991; Meyer et al., 1992; reviewed by Fu & Gordon, 1997). Therefore, it seems that, besides phagocytosis of myelin and axonal debris, the interactions of SCs with infiltrated macrophages during Wallerian degeneration may be essential for the maintenance of the growth-supportive phenotype of the SCs.

Transforming growth factor-β (TGF-β) is a versatile cytokine that modulates SCs growth and differentiation, and it is secreted by both SCs and macrophages during Wallerian degeneration (Assoian et al., 1987; Ridley et al., 1989; Constam et al., 1992; reviewed by Unsicker & Strelau, 2000). Its secretion may be mediating the sustenance of the growth-supportive non-myelinating phenotype of the SCs during Wallerian degeneration. Therefore, experiments were designed to test whether exposure of chronically denervated SCs to TGF-β in vitro would be able to induce their growth-supportive phenotype, and improve their capacity to support axonal regeneration in vivo. Indeed, TGF-β was able to improve the capacity of chronically denervated SCs to support motor axonal regeneration (Sulaiman & Gordon, 2001a). The results of these experiments further suggest that Wallerian degeneration is essential for the reparative process that ensues after peripheral nerve injuries, although the cellular-molecular events of Wallerian degeneration remain to be fully elucidated.

7.1.1.2 Chronic Neuronal Axotomy

Injured motoneurons upregulate neurotrophic factors and other regeneration-associated genes (RAGs) that support regeneration of their peripherally injured axons. However, the expression of these RAGs is very transient (reviewed by Fu & Gordon, 1997), such that further regeneration of axons by motoneurons, especially over a long distance, is dependent on quick access to target- and SC-derived RAGs by regenerating axons. If nerve repair is delayed or as a result of slow rate of regeneration over a long distance, injured motoneurons remain chronically axotomised, and by the time their axons reach the distal nerve stumps, the expression of RAGs in the SCs which remain denervated for a long time, may have been downregulated (You et al., 1997). Therefore, regeneration of axons by injured motoneurons at a faster rate is warranted to prevent chronic axotomy of the injured motoneurons, and loss of the expression of RAGs by SCs as a result of chronic denervation.

7.1.1.2.1 FK506 Can Prevent Chronic Neuronal Axotomy and Schwann Cell Denervation by Increasing Rate of Axonal Regeneration

The immunophilin FK506 has been shown to accelerate motor axonal regeneration in rat models of crush, and transection injuries that have been primarily repaired (reviewed by Gold et al., 2000). However, clinical experience has shown that most nerve injuries require either delayed primary repair or secondary repair because patients with injured nerves often have multiple traumas and the wound sites are often too contaminated to be repaired primarily. Under these circumstances, injured motoneurons remain chronically axotomised and the SCs in the distal nerve stumps remain chronically denervated, pending nerve repair. Therefore, we tested the efficacy of FK506 in experimental models of nerve injury and repair that mimic the clinical scenarios, that is, after chronic axotomy of motoneurons, and chronic denervation of the SCs. We found that FK506 significantly increases the rate of axonal regeneration by motoneurons despite the fact that they have been subjected to chronic axotomy but, it does not improve motor axonal regeneration into chronically denervated SCs (Sulaiman et al., 2001b). However, the use of FK506 to accelerate motor axonal regeneration would prevent both chronic motoneuronal axotomy and chronic SC denervation since motoneurons that regenerate their axons at a faster rate would reinnervate the SCs of the distal nerve stumps sooner, and prevent their chronic denervation. The use of FK506 in the clinic for the sole purpose of accelerating motor axonal regeneration is yet to be fully investigated.

7.1.1.3 Misdirection of Axonal Regeneration

Another major obstacle to optimal functional recovery after nerve injuries is the misdirected regeneration of axons into the wrong endoneurial tubes, For example, regeneration of motor axons into endoneurial tubes that direct them to reinnervate the skin. Axons which regenerate in the wrong endoneurial tubes are lost and are functionally useless.

7.1.1.3.1 Sensory Endoneurial Pathways Play an Active Role in Preferential Motor Reinnervation

Axonal regeneration of injured femoral nerve, which is composed of completely divided motor and sensory braches, is unique. This is because after femoral nerve injury, misdirected regeneration of axons is limited since regenerating motor axons preferentially reinnervate the distal nerve stump of the motor nerve. This phenomenon is termed preferential motor reinnervation and its occurrence has been linked to the expression of specific adhesion molecules by SCs of the motor endoneurial tubes, which preferentially attract regenerating motor axons (e.g. HNK-1; Martini et al., 1994). The contribution of the sensory endoneurial to preferential motor reinnervation is not well understood.

Results of our experiments designed to promote motor axonal regeneration in a long sensory autograft by neurotising one end of the autograft with a sensory nerve while a motor nerve regenerate into the other end of the autograft, showed that the presence of the sensory nerve actually reduced motor axonal regeneration, contrary to our expectations (Sulaiman et al., 2001c). In addition, in the presence of the sensory nerve, SCs of the sensory nerve graft failed to maintain the integrity of regenerated axons, and remyelinated regenerated axons very poorly. Therefore, it seems that the presence of sensory axons preferentially induces some changes in the sensory SCs which makes them less supportive to regenerating motor axons, especially since neurotisation of the autograft with a motor nerve promoted motor axonal regeneration and, re-myelination of regenerated axons by the sensory SCs (Sulaiman et al., 2001c) . These somewhat specificity in the interactions of SCs with regenerating axons suggest that the emergence of preferential motor reinnervation after femoral nerve injury, is subsequent not only to the neurotropic effects of the SCs in the motor endoneurial tubes on regenerating motor axons, but also as a result of the 'repulsive' effects of the SCs in the sensory endoneurial tubes on the regenerating motor axons.

7.2 CONCLUSION

The findings of this thesis demonstrated, using a direct neuroanatomical estimate of motoneuronal regeneration, that 1) chronic SC denervation detrimentally affects their capacity to support axonal regeneration by injured motoneurons (Sulaiman & Gordon, 2000); 2) the capacity of chronically denervated SC to support motoneuronal regeneration can be improved by application of exogenous cytokines such as TGF-β Sulaiman & Gordon, 2001a; 3) FK506 accelerates motor axonal regeneration by chronically axotomised motoneurons, and by so doing, can avert the deleterious effects of the slow rate of regeneration by injured motoneurons (chronic axotomy and denervation) on functional recovery Sulaiman et al., 2001b; 4) the responsiveness of sensory SCs to regenerating axons can be specifically primed to be more or less responsive to motor nerve, depending on whether they are interacting with a another motor or sensory nerve, respectively (Sulaiman et al., 2001c).

7.2.1 Multiple Approaches Are Required to Optimise Functional Recovery after Peripheral Nerve Injuries

Chronic SC denervation, chronic motoneuron axotomy and misdirection of regenerated axons are 3 main factors that could detrimentally affect functional recovery after microsurgical repair of injured peripheral nerves (Fu and Gordon, 1997). This thesis examines the 3 factors, although to different extents, and reports experimental strategies that reverse the detrimental effect of chronic denervation on SCs and chronic axotomy on motoneurons. It also sheds more light on the possible role of sensory SCs in promoting preferential motor regeneration, which has been previously reported to be promoted by functional electrical stimulation (Al-Majed et al., 2000). The use of neurotrophic factors, BDNF and GDNF to promote motor axonal regeneration has also been reported by several authors, including our laboratory (Boyd & Gordon, 2000). However, the combined effects of the experimental strategies outlined in this thesis were not investigated, and form part of the experiments to be carried out in the future. Likewise, experiments which examine the combined effects of functional electrical stimulation and the strategies addressed in this thesis or application of neurotrophic factors, may determine additive effects of all these strategies. Therefore, it is essential to realise that multiple experimental approaches may be required to optimise motor axonal regeneration and functional recovery after peripheral nerve injuries.

7.3 FUTURE DIRECTIONS

In this thesis, I argue that SC-macrophage interactions during Wallerian degeneration is essential for the maintenance of the growth-supportive phenotype of the SCs. This argument was substantiated by the fact that, TGF-β, a cytokine that is secreted during Wallerian degeneration by both SCs and macrophages, increased the capacity of chronically denervated SCs to support motor axonal regeneration. However, further experiments are required to understand the mechanism of this effect of TGF-β on chronically denervated SCs. The experiments outlined below may be able to answer this question:

7.3.1 Investigation of the Regulation of the Growth-supportive Phenotype of the Schwann Cells During Wallerian Degeneration by Cytokines *in Vitro*

The effects of TGF-β on chronically denervated SCs that made them more supportive of motor axonal regeneration in vivo, can be elucidated by applying TGF-β to SCs that have been isolated from chronically denervated nerve stumps in vitro. Following incubation with TGF-β for 48 hours, changes in these SCs, as far as phenotype and proliferation are concerned can be assessed either by immunocytochemistry, western blot or RT-PCR. The effects of other cytokines such as IL-1, TNF-α, IL-6 and IL-10 which have also been associated with Wallerian degeneration, could be examined in this same manner as well.

7.3.2 Application of Delineated Cytokines *in Vivo* to Maintain Schwann Cell Growth-supportive Phenotype

The above experiments would shed more lights on the molecular regulation of SC growth, and their growth-supportive SC phenotype by cytokines. They would demonstrate which cytokines promote the growth-supportive phenotype that can possible be used to maintain the phenotype in vivo, although further titration of the doses of cytokines to be used ma y be required. Delineated cytokines from the groups which promote SC growth-supportive phenotype are potential pharmacological tools that could be used in the clinic either locally or otherwise, to preserve the distal stumps of injured nerves pending microsurgical nerve repairs.

7.3.3 Investigation of the Combined Effects of Cytokines and Fk506 on Peripheral Nerve Regeneration and Functional Recovery

Cytokines which promote the growth-supportive phenotype of SCs may be used in combination with subcutaneous injection of FK506, which overcomes the adverse effects of chronic axotomy on injured motoneurons (Sulaiman et al., 2001c). This effect of FK506 also presents a very useful tool that could be used to promote functional recovery in patients who have suffered peripheral nerve injuries, especially since its immunosuppressive effect is not elicited at the dose used to promote nerve regeneration in our and other experiments (reviewed by Gold, 2000).

7.4 REFERENCES

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